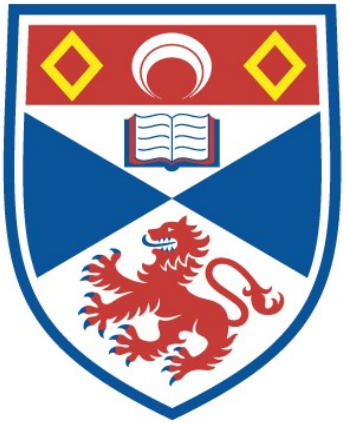
THE MECHANISMS AND CONSEQUENCES OF OVIPOSITION DECISIONS IN THE EUROPEAN BITTERLING

André Phillips

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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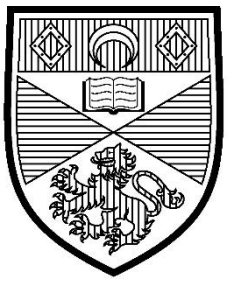

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Abstract

Oviposition-site decisions can have disproportionate effects on offspring survival and success, and while the effects of these decisions are frequently investigated, the processes underpinning these decisions and the cues used are often less well understood. The aim of this thesis was to understand the mechanisms associated with making oviposition decisions, and their consequences, using the European bitterling (*Rhodeus amarus*), a small freshwater fish that lays its eggs in the gills of freshwater mussels.

Using an artificial mussel, females were shown to respond to dissolved oxygen, relating to offspring survival, while males attend to water flow velocity, with implications for sperm competition. Oviposition decisions by either sex were unaffected by olfactory cues associated with sperm release, though males and females were responsive to visual cues associated with ejaculation. An analysis of the placement of eggs within the mussel gills by females failed to show evidence of "handedness", though there was a tendency to place eggs in a mussel's inner gills, which may be adaptive in avoiding competition with the mussel's embryos. Male response to oviposition sites showed inter-population variation, with males from a population with high levels of mean crowding showing an elevated frequency of mussel inspection and probability of ejaculation. Males also increased the frequency of mussel inspection in response to the presence of a gravid female, suggesting a role for sperm competition and fertility assurance. Social network analysis was used to investigate how male interactions affect oviposition decisions. This approach showed that male mating tactics could be understood by quantifying how males distributed their sperm among oviposition sites. Findings are discussed in the context of our understanding of the bitterling mating system and research on oviposition-site decisions.

Acknowledgements

First and foremost, thanks and respect go to my supervisor Carl Smith for constant support, assistance and guidance from taking on a naïve 2nd year undergraduate as a summer research volunteer all the way through to the completion of this PhD. Second thanks has to go to Martin Reichard of the Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic. Without his assistance in brainstorming, extensive facilities and fieldwork, this research would not have happened. Others at the Brno institute also deserve immense thanks in this respect and for happily answering the day-to-day questions of fish collection, care and research: Matej Polačik, Radim Blažek, Milan Vrtílek and many others. Thanks also go to Ali Serhan Tarkan, Özcan Gaygusuz and Ertan Ercan of the Fisheries Faculty of Istanbul University for assistance with fieldwork in Turkey. Rowena Spence deserves special mention for assisting with data collection and reviewing manuscripts. Thanks also goes to the other PhD students in 'the Call Centre' office, especially Helen Spence-Jones, Andrew Clark, Kat Falkenberg, Rob Patchett, Viviana Brambilla and Jess van der Wal for the excellent support network, stats, rants and snacks.

Any work needs a life balance to it. St Andrews University Canoe Club has been my family while at university and kept my sanity through the years, particular mention to my academic family Freja Larsen, Jenna Earp, Andrew Hunter, Hugo Fairclough and Kathryn Haddick for the great moments and moments when sanity was not wholly kept.

Contributions

The thesis was written and the majority of fieldwork conducted by myself, but the specific assistance by others is outlined here. All chapters were written by myself with draft assistance from Carl Smith & Helen Spencer-Jones. The artificial mussel used in Chapter 2 was developed by myself from a design by Dr Yoshihiko Kanoh and his students at Seifu High School, Osaka, Japan. Throughout the thesis, fieldwork logistics, materials, and advice were provided by Martin Reichard at the Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic. Assistance with collection of fish and additional advice provided by Matej Polačik, Radim Blažek and Milan Vrtílek through all the field seasons. Chapter 5 fieldwork assistance in Turkey was provided by the Fisheries Faculty of Istanbul University in the form of accommodation, a lab and assistance collecting fish. In Chapter 6, Carl Smith and Rowena Spence assisted in collection of data.

Contents

Figures and Tables

PREAMBLE

In oviparous taxa, oviposition-site decisions can represent a critical feature of the mating system. In species that do not perform parental care, oviposition-site decisions are the primary means by which a parent, usually the mother, can influence the fitness of offspring, particularly if oviposition sites vary in quality. Choice of oviposition sites can have implications not just for individual reproductive success but also for population processes (Refsnider & Janzen, 2010).

Several invertebrate model systems have been used to understand the implications of oviposition-site decisions, including parasitoids (Taylor et al., 1998; Godfray, 2004), seed beetles (Cope & Fox, 2003) and phytophagous insects (Mayhew, 1997). While oviposition-site decisions are recognised as critical for vertebrates, they have been less well studied (Refsnider & Janzen, 2010).

Here, a species of teleost fish with a well-characterised spawning behaviour is used, to understand the proximate mechanisms underlying oviposition-site decisions in vertebrates. Bitterling fishes use freshwater bivalve mussels as sites for oviposition and offer a model system for understanding the implications of oviposition-site decisions. While features of the mating system of these fishes have been studied, including sperm competition, coevolution, sexual conflicts and alternative mating tactics (Reichard et al., 2004a; Casalini et al., 2009; Reichard et al., 2010; Smith et al., 2014a,b), the proximate mechanisms for selecting oviposition sites are disputed (Smith et al., 2004). Understanding the mechanistic basis to oviposition-site decisions is critical to recognising plasticity in this behaviour, its significance in mating system evolution, and how oviposition-site decisions may vary in response to environmental change or degradation. While there are approximately 100 species of bitterling fish, primarily in

East Asia (Chang et al., 2014), this thesis will focus on the best-studied species, the European bitterling (*Rhodeus amarus*). Where relevant, reference is made to other bitterling taxa. While relatively few bitterling species have hitherto been the subjects of experimental behavioural studies, where comparative studies have been undertaken *R. amarus* appears to express a generalised mating system that is broadly applicable to related species (e.g. Reichard et al., 2007a; Pateman-Jones et al., 2011; Smith et al., 2014a).

ORGANISATION OF THE THESIS

This thesis is organised into seven chapters, each addressing a different aspect of oviposition-site decision making in bitterling. In **Chapter 1,** a comprehensive summary of the current state of knowledge of the bitterling mating system is given, providing context for experimental studies presented in **Chapters 2-6**, and making specific reference to the role of oviposition-site decisions in the mating system. Using an artificial mussel host, the results of an experimental study is presented in **Chapter 2** to identify the cues used by male and female bitterling in host choice decisions. In **Chapter 3**, the cues used by bitterling for oviposition choice are further investigated, focusing on the role of sperm as a cue for assessing fertility assurance and sperm competition in females and males respectively. In **Chapter 4,** whether individual preferences for left or right oviposition, i.e. 'handedness', plays a role in how female bitterling distribute their eggs in the gills of hosts mussels is investigated, while in **Chapter 5**, male host preferences in response to the presence of a rival male or spawning female are examined, addressing variation among populations and considering the significance of multiple ejaculations in the mating system. The

approach to understanding oviposition-site decisions in bitterling is expanded in **Chapter 6**, using social network analysis for data collected in a semi-natural spawning population. Finally, in **Chapter 7**, the implications of the findings are considered for the bitterling mating system and for understanding oviposition-site decisions more generally.

1.0 Chapter One

THE MATING SYSTEM OF THE BITTERLING FISHES

1.1 ABSTRACT

Bitterling fishes have been used as a model species to study a range of questions in biology relating to both mating system evolution and coevolution. Males defend territories containing freshwater mussels and lead females to the mussels. Female bitterling oviposit eggs within the gills of freshwater mussels, where the embryos complete development. Bitterling display a wide range of adaptations to their unusual mode of reproduction. These include morphological adaptations such as specialised ovipositors and sperm but also behaviours such as multiple ejaculation, skimming and alternative mating tactics. Because they use a spawning site that can be readily manipulated and assessed for quality, bitterling are an unusually tractable system for researching oviposition and mate choice decisions. This chapter reviews the mating system of the bitterling, focussing on the best-studied species, the European bitterling (*Rhodeus amarus*), with reference made to other bitterling species where relevant. The review identifies behavioural decisions associated with oviposition-site decisions as a central component of the mating system. This is due to the strong effect of host choice on offspring fitness. This review proposes further research into the oviposition decisions of the bitterling, including the experimental analysis of host cues such as oxygen, flow and presence of sperm. The social cues experienced by bitterling and how they may affect host choic are also suggested as a further route for study.

1.2 INTRODUCTION

Bitterling (Cyprinidae, Acheilognathinae) are small teleost fishes found in shallow freshwater lakes, ponds, rivers and streams across Europe and East Asia (Duyvené de Wit, 1955; Wiepkema, 1961; Smith et al., 2004). Bitterling have an unusual mode of reproduction. During the spawning season, male bitterling guard territories around freshwater bivalve mussels and engage in active courtship of females, which they lead to mussels. Females possess an unusually long ovipositor which lengthens after the female ovulates a batch of eggs and is ready to spawn. With this ovipositor, the female is able to place a clutch of eggs inside the mussel's gills by inserting it into the exhalent siphon. Male bitterling fertilise the eggs by repeatedly releasing sperm over the inhalant siphon of the mussel, which is drawn into the gill by the flow of water generated by the mussel for feeding and respiration. No parental care is provided by either parent and the embryos remain inside the mussel gill for between 2-4 weeks (depending on water temperature), until the yolk-sac is exhausted (Smith et al., 2004; Liu et al., 2006). Embryos eventually emerge from the mussel as well-developed larvae.

1.2.1 Purpose and scope of the review

The goal of this review is to provide an overview of the current understanding of the mating system of the bitterling fishes, while building upon and updating, the reviews by Wiepkema, (1961) and Smith et al., (2004), both of which focused primarily on the biology of the European bitterling, *Rhodeus amarus*. Bitterling-mussel coevolution, which has been a theme of much recent research on bitterling fishes (Reichard et al., 2010), will be dealt with briefly, and only in the context of mating system evolution.

While including published information on all bitterling species, the review necessarily focuses chiefly on two species; *R. amarus* and *R. ocellatus* (Chinese rose bitterling), reflecting the preponderance of published studies on these two species. Where appropriate, studies on other species will be discussed and interspecific differences highlighted. The review will particularly focus on research areas that are of current interest and will highlight gaps in understanding of bitterling reproductive biology.

The review first addresses the history of bitterling research and phylogenetic relationships of bitterling species. The relationship with the mussel hosts is discussed followed by the anatomy of bitterling in general. The courtship and mating behaviour of male and female bitterling are considered, and the significance of alternative mating tactics, sperm competition, host choice and offspring development discussed. The review concludes by identifying areas of research on the mating system that warrant further investigation.

1.2.2 Bitterling as model species for mating system evolution

Model organisms further the knowledge of general patterns in biology and provide insights into other species. Bitterling represent an excellent model species on account of the notable physiological, morphological and behavioural adaptations of their unusual mode of reproduction, allied with the ability to quantify and manipulate the spawning site. Bitterling also exhibit a range of striking behaviours, including male-male competition, alternative mating tactics and mate choice. Due to these attributes, bitterling are an excellent study species for investigating a range of aspects of behavioural biology. These include the study of host choices in an ovipositing species,

mate choice (due to the disassociation of mate and host choice in bitterling), sperm competition and host-parasite interactions.

Hettyey et al. (2010) outlined the features of an ideal species for studying mate choice; external fertiliser, no parental care and no recorded cryptic female choice, all of which are features of bitterling (Smith et al., 2004). Bitterling are particularly amenable to experimental study. They are easily observed in their natural environment and in aquaria, are resistant to temperature changes, disease and spawn many times a day over a breeding season. Bitterling also perform discrete, unambiguous behaviours, making them ideal subjects for behavioural observation. As a specious group with a wide distribution across Asia and Europe, there are many opportunities for interspecific comparisons. Some bitterling species are facing extinction (Chen & Yang, 2008). The study of related bitterling species allows a better understanding of the biology which could provide essential information for the conservation of endangered species without impact to at-risk species. In other regions of the bitterling's range, they are invasive, allowing the study of recent sympatry interactions. Finally, the phylogenetic relationships of bitterling are well characterised (Chang et al., 2014; Kawamura et al., 2014), an essential requirement for evolutionary comparative studies (Felsenstein, 1985). Overall, the bitterling represents an unusually tractable model for behavioural and evolutionary studies focused on mating system evolution, sexual conflict and, owing to their relationship with mussel spawning hosts, coevolution.

1.2.3 A brief history of research on bitterling fishes

The first record of finding fish eggs and larvae in the gills of freshwater mussels was that of a German physician, Döllinger, in 1818 (Döllinger, cited in Noll, 1869).

However, it was several decades later that the source of these eggs was identified to be that of European bitterling (Noll, 1869). In Europe, early research on *R. amarus* focused on its use as a human hormonal assay for pregnancy (Fleischmann & Kann, 1932; Ehrhardt & Kuhn, 1933), though its value for this purpose proved limited (Duyvené de Wit, 1955). The first systematic study of bitterling in Europe was conducted by Holčík in Czechoslovakia, who focused on its taxonomic position, population dynamics and life history (Holčík, 1959). In collaboration with Duyvené de Wit, Holčík also undertook hybridisation experiments with Asian bitterling species (Holčík & De Wit, 1962). The first major behavioural work was that of Wiepkema in Holland, who conducted a detailed ethological study of *R. amarus*, a large component of which was focused on reproductive behaviour (Wiepkema, 1961). Little further systematic work was performed on *R. amarus* until the end of the 20th Century when Reichard at Masaryk University in the Czech Republic (Reichard, 1996) and Aldridge at Cambridge University in the UK (Aldridge, 1997); both independently completed theses on the biology of the species. Subsequent work in the UK (Reynolds) and continental Europe (Reichard, Smith), addressing behavioural and evolutionary questions, demonstrated the value of the species for experimental research on mate choice (Mills & Reynolds, 2002a; Smith et al., 2004; Reichard et al., 2004a). Subsequent research by Smith and Reichard further addressed bitterling-mussel host-parasite coevolution (Reichard et al., 2010) as well as mating system evolution (Reichard et al., 2009)

In Asia, where bitterling show their greatest species diversity, research on the biology of bitterling has been more systematic and driven by the conservation research of endangered species and sub species (Kawamura et al., 2014). The taxonomy and phylogenetic relationships of bitterling have been comprehensively described by

Okazaki et al. (2001) as has the genetic diversity of endangered species (Kubota & Watanabe, 2003; Kawamura et al., 2014). Developmental biology, particularly of Japanese species, has been addressed, beginning with work by Nagata (Nagata, 1985), and included studies by Kanoh (Kanoh, 2000), and Kawamura (Kawamura & Uehara, 2005), with a recent expansion in studies of this nature (Kitamura, 2005; 2007, Nishio et al., 2015). In China the focus of research has been taxonomic and phylogenetic, notably work by Liu (Liu et al., 2006) and Li (Li & Arai, 2010; 2014; Li et al., 2015) as well as some behavioural studies (Wang et al., 2013). Research on Asian species has also tackled reproductive cues (Kitamura, 2007) and embryo development (Kitamura, 2005).

1.3 TAXONOMIC STATUS

The bitterling fishes are grouped within the cyprinid sub-family Acheilognathinae, comprising three genera, *Acheilognathus*, *Rhodeus* and *Tanakia* (Nelson, 2006). Two additional genera have been proposed by both Bohlen et al. (2006) and Chang et al. (2014) (*Pseudorhodeus* and *Paratanakia*). Several studies have further proposed that the Acheilognathinae be elevated to family level, given that they represent a highly speciose monophyletic clade in the Cyprinidae (Saitoh et al., 2006; Chen & Mayden, 2009; Chang et al., 2014).

Almost all published behavioural and evolutionary studies have hitherto focused on *Rhodeus* spp., with little work on other genera and with the majority of studies conducted using the European bitterling (*R. amarus*). Two additional species, *R. meridionalis* (Bohlen et al., 2006) and *R. colchicus* (Bogutskaya & Komlev, 2001) have been described in Europe, though their status remains disputed. Phylogenetic analysis by Bryja et al. (2010) supported the divergence of *R. meridionalis*, while *R. colchicus*

displayed mixing of haplotypes with *R. amarus* and, in this study at least, its status as a separate species was not strongly supported. More recent data supports six major lineages within the distribution of *R. amarus* (Danube Basin, western Black Sea, eastern Black Sea, Caspian Sea, western Aegean Sea, eastern Aegean Sea), and these recent data suggest that *R. amarus* is probably best considered as a species complex (Martin Reichard, unpublished data). Similar patterns may emerge in other widespread bitterling taxa in Asia, such as *R. ocellatus* (Huanzhang Liu, unpublished data).

1.4 MUSSEL HOSTS

Instead of performing potentially costly nest building, parental care and clutch defence (Smith & Wootton, 1995), bitterling utilise a range of freshwater mussel species as hosts which act as incubators for their eggs and embryos. Hosts belong primarily to the Unionoida (Reichard et al., 2007a), but the Margaritiferidae (Smith & Hartel, 1999) and Cyrenidae (Li et al., in press) mussel groups are also reported to be used by Asian bitterling species. Freshwater mussels filter water, driven by cilia action in their gills, to obtain oxygen and food items and to remove metabolic waste. The gill of these mussels consists of four lamellae structures made up of water tubes that are arranged in two bi-symmetrical sets of inner and outer demibranchs. Water enters the mussel through the inhalant siphon into the water tubes in the gill lamellae and it is here which both bitterling and mussel embryos are incubated. Gill complexity among mussel species varies, from a relatively simple gill structure in the Margaritiferidae with gills lacking true water tubes, to the more complex tripartite water tubes of mussels in the Anodontinae in the Unionoida (Wu, 1998). Greater complexity of the gill correlates

with higher oxygen consumption (Wu, 1998), with implications for bitterling embryo development.

1.4.1 The Parasite-Host Relationship

Mussels reproduce by releasing glochidia; small obligate parasitic larvae that attach to fish gills and fins, encyst and break down tissue for nourishment. Glochidial infection can cause an increase in fish mortality (Myers & Millemann, 1977). It was formerly proposed that the relationship between bitterling and mussels represented a mutualism, with mussels hosting bitterling embryos and bitterling hosting mussel glochidia (Wheeler, 1978). Reichard et al. (2006) tested the fitness consequences of the relationship between bitterling and mussels by recording glochidia attachment rates to five non-bitterling fish species, including *R. amarus* and *R. ocellatus*. They also examined the growth rate of *Unio pictorum* with high or low numbers of bitterling embryos in their gills. They found that both *R. amarus* and *R. ocellatus* had reduced glochidia infection compared to other fish species. Reduced glochidia infection rates in *R. amarus* were also demonstrated by Dudgeon & Morton (1984) and Aldridge (1999). Both Aldridge (1999) and Reichard et al. (2006) showed that five days after initial infection, glochidia detached from *R. amarus* and *R. ocellatus* suggesting an immune rejection of glochidia by bitterling. Immune responses to glochidia have been demonstrated in European chub (*Squalius cephalus*) (Douda et al., 2016) and bluegill sunfish (*Lepomis macrochirus*) (Rogers & Dimock, 2003). It is likely that bitterling have an immune response to glochidia (Reichard et al., 2006), though the mechanism remains untested and it is unclear if the response is innate or acquired. Reichard et al. (2006) also showed that *U. pictorum* displayed a significant growth reduction when bitterling embryo infection rates were high. Bitterling infection likely reduced growth

through competition for oxygen, reduced gill efficiency and competition for amino acids (Stadnichenko & Stadnichenko, 1980; Reichard et al., 2006; Spence & Smith, 2013). Bitterling benefit from the relationship at a cost to the host mussels and hence the relationship between bitterling and mussel can be considered as a parasitic relationship (Connor, 1995).

In areas of ancient sympatry between bitterling and mussels it appears that mussels have evolved defences against bitterling parasitism (Reichard et al., 2010; 2015). These defences involve ejection of bitterling eggs by mussels through rapidly closing their siphons and contracting their valves to force eggs and embryos out of their gills (Reichard et al., 2007b; 2010). Bitterling show a number of counter-adaptations which ensure egg and embryo remain lodged in the mussel gills (Smith et al., 2000a; Mills & Reynolds, 2002a; Kitamura et al., 2012; Rouchet et al., 2017) (see Embryo Adaptations).

1.5 MORPHOLOGY & APPEARENCE

Most bitterling species are small, typically less than 70 mm standard length (SL), with a laterally compressed body shape. However, there is variation in size among species, with smaller species, such as *R. fangi*, growing to a maximum SL of approximately 50 mm and larger species, such as *A. macropterus* up to 250 mm (Nichols, 1943).

Among species, bitterling display a range of colouration, some of which appears to function in mate choice. Most bitterling display a pale silver colour ventrally and a darker grey-green dorsally. During the spawning season males display striking nuptial colouration, particularly on the fins, but also on the body, on the lips and in the iris (Smith et al., 2014b). *R. amarus* and *R. ocellatus* expresses red and black markings on

dorsal and anal fins with a red iris. In the related Chinese bitterling, *R. sinensis*, nuptial colouration is predominantly black and orange. In some species, particularly those associated with lake habitats, such as *A. tabira tabira* and *A. melanogaster*, nuptial colouration predominantly comprises black and white patterns (T. Cain, unpublished data), possibly relating to light attenuation at greater depths. Nuptial colouration and indirect benefits of mate choice are often linked (Andersson & Simmons, 2006) but colour can also signal direct benefits, such as efficiency of fertilisation (Pike et al., 2009). Carotenoid-based colours, such as red, orange and yellow, appear frequently in bitterling colouration and may function as sexual signals (see Mate choice below).

1.6 REPRODUCTIVE BEHAVIOUR

1.6.1 Territoriality

The mating system of bitterling is ostensibly a form of resource-defence polygynandry, based around males controlling access to mussel oviposition sites, which females visit to spawn. Resource guarding by guarder males reduces the risk of sperm competition (see Alternative mating tactics and sperm competition below) by driving away rival males, with guarders often achieving the highest reproductive success (Birkhead & Møller, 1998; Reichard et al., 2004a; 2005). Male bitterling establish territories around mussels, which vary in size according to male dominance and rival male density (Smith et al., 2004). Male dominance is determined chiefly by body size but additionally by iris colour area and intensity in *R. amarus* and *R. ocellatus* (Kanoh, 2000; Smith et al., 2002; Reichard et al., 2005; 2009; Casalini et al., 2009). Smith (2011) showed that male *R. ocellatus* utilise mutually-agreed territorial boundaries, thereby reducing the costs of aggression and display behaviours. Territories that contain more mussels are more

likely to attract females (Przybylski et al., 2007), though mussel quality and interference by rivals also play a role (Smith et al., 2002). Male bitterling make complex mating decisions, switching between guarder and sneaker roles, though the contexts in which these decisions are made are currently poorly understood.

Guarder males aggressively defend territories from rivals, with escalation in behaviour from fin spreading and parallel swimming, behaviours that signal size and enable males to evaluate rivals to directly aggressive behaviours (Wiepkema, 1961). Male conflict can include a 'jerking' behaviour, striking the other male with their body or fin or head-butting one another. At high male densities, territoriality breaks down and becomes group spawning (Kanoh, 2000; Reichard et al., 2004a).

1.6.2 Courtship

When a female with an extended ovipositor approaches the territory of a guarder male he will engage the female in courtship behaviour and attempt to lead her to a mussel. Males display to the female by fin-spreading and performing rapid body undulations termed 'shivering' (Wiepkema, 1961). The male may also swim rapidly back and forth between the female and the mussel. Particularly vigorous male courtship attracts females and may increase the likelihood of spawning (Reichard et al., 2005; Casalini et al., 2009). Male colour has been shown to increase female attention to male leading behaviour (Candolin & Reynolds, 2001; Jeon & Suk, 2012), however other cues associated with male quality and mussel quality play a role in female spawning decisions (see Mate choice and Oviposition decisions below)

1.6.3 Mate choice

Female bitterling appear to show mating preferences for specific males (Casalini et al., 2009). The basis of these mate choice decisions has been the focus of several studies. Fitness benefits of mate choice can be described as either direct or indirect (Trivers, 1972; Andersson, 1994; Neff & Pitcher, 2005). Direct fitness benefits improve the fitness of the female or the offspring through non-genetic means, such as direct provisioning or fertility assurance (Andersson, 1994). Indirect benefits affect the offspring through genetic mechanisms, either additive 'good genes' effects or non-additive compatibility (Neff & Pitcher, 2005).

Fertility assurance is an important direct benefit for females (Levitan, 1998). Sperm depletion (Nakatsuru & Kramer, 1982) or male reduction in the frequency of sperm release (Pateman-Jones et al., 2011) are both relevant in the context of the bitterling mating system, particularly for successful males who have an increased risk of sperm depletion. In *R. amarus*, a solitary male sometimes fails to fertilise all the eggs a female produces in a spawning event (Smith & Reichard, 2005; Smith et al., 2009) and during pair spawning it is predicted that females should select males who will increase fertility assurance of their eggs. Larger males produce more sperm (Smith et al., 2014b) and also experience fewer interruptions during courtship (Smith et al., 2002).

Colouration is carotenoid-based in many bitterling species and these compounds can function in signalling parasite resistance, growth, foraging ability and overall condition (Olson & Owens, 1998; Chew & Park, 2004). Carotenoid-based colouration is common in other fishes, such as the three-spined stickleback (*Gasterosteus aculeatus*) (Pike et al., 2009) and guppy (*Poecilia reticulata*) (Locatello et al., 2006). In a study of *R. amarus* males, carotenoid colouration was a highly significant predictor of spermatozoa numbers in the testes, with approximately 26% of variance in the number

of spermatozoa stripped from males predicted from male colour after controlling for male body size. Thus, in this species at least, the extent of male nuptial colouration may signal a direct benefit to female of fertility assurance (Smith et al., 2014b).

Heritability studies are necessary to distinguish whether indirect benefits of mate choice are through good genes or compatibility. Good genes selection predicts that females will select for heritable traits in males and that are linked to improved fitness irrespective of female genetic background (Kempenaers, 2007). Compatible genes selection predicts that both the male and female haplotype are relevant. Using *R. ocellatus*, Agbali et al. (2010) conducted a fully crossed heritability study which demonstrated significant female additive effects on offspring fitness through egg size, but no male additive effects. However, they did show strong non-additive compatibility effects.

The compatibility model of indirect mate choice benefits predicts that parental genes will produce offspring of higher fitness though non-additive benefits (Zeh & Zeh, 1996; Neff & Pitcher, 2005). Compatibility has been studied as a mechanism for the evolution of polyandry, particularly in birds (Slagsvold & Lifjeld, 1994). If mate choice follows the compatibility model, mate choice will be non-congruent, indicating that each male varies in quality according to the female's genes. The benefits of compatibility will be most visible as resistance to parasites and disease (Tregenza & Wedell, 2002; Garant et al., 2005).

Major Histocompatibility Complex (MHC) alleles have been implicated as the mechanism for indirect mate choice decisions in *R. ocellatus* (Agbali et al., 2010; Reichard et al., 2012). MHC genes play a role in the transport of foreign peptides to the cell surface for display to the immune system. The greater range of MHC alleles an

organism has, the more foreign peptides can be recognised by the immune system and the more effective the immune response. Each parasite is only identified by the immune system by 1 or 2 peptides so a wide range of MHC alleles is essential. MHC alleles can influence individual odour (Brown, 1995; Yamazaki & Beauchamp, 2007), and this has been suggested as a mechanism for detecting genetic compatibility (Milinski, 2006; Yamazaki & Beachamp, 2007; Setchell et al., 2010). Female *R. ocellatus* were shown to prefer spawning with MHC dissimilar males and chose non-congruently (Agbali et al., 2010; Reichard et al., 2012), though whether the same mechanism operates in other bitterling species is not yet known.

1.6.4 Alternative mating tactics and sperm competition

Reproductive behaviour can vary between members of the same sex and a variety of alternative mating tactics have been described in a range of taxa (Birkhead & Møller, 1998). Alternative mating tactics are often associated with sperm competition (Shuster, 2010). Sperm competition describes competition between the sperm of two or more males for the fertilisation of an egg (Parker, 1970). Sperm competition is described in terms of sperm competition *risk* and *intensity*. The *risk* of sperm competition is the probability that a male's sperm will compete with the sperm of other males. The *intensity* of sperm competition is the extent of overlap of the ejaculates of different males and, therefore, the number of males that engage in sperm competition at a mating and the quantity of sperm they contribute to a mating (Parker, 1998; Wedell et al., 2002). In bitterling, territorial defence of a mussel in combination with courtship of females is just one tactic males can adopt to achieve reproductive success. Sneaking is an alternative tactic to guarding whereby the males do not defend territories or court

females, but instead attempt to obtain matings without any associated costs (Andersson, 1994). Sneak mating tactics have been observed in *R. amarus*, *R. ocellatus* and, *R. sinensis* (Smith et al., 2004; Reichard et al., 2004a; Pateman-Jones et al., 2011). In these species, both before and after the female spawn, a sneaker may quickly swim above the mussel and release sperm, attempting to gain a proportion of paternity of the eggs released by the female. Male mating tactics in bitterling are conditional (*sensu* Gross, 1996), with a male able to switch between different tactics with no distinct phenotype associated with either tactic (Kanoh, 2000; Smith et al., 2004). Larger males tend to be guarders as dominance is determined by size. Large territorial males will often still sneak neighbouring mussels while defending their own territory. A third tactic, termed 'pirate' (van den Berghe, 1988; Mboko & Kohda, 1999) has also been suggested for bitterling whereby males take over the mussels of rivals for a short time when a female spawns, before subsequently moving on to another mussel.

Surprisingly, the timing of sperm releases prior to oviposition (pre-oviposition ejaculation) is the best predictor of male fertilisation success in *R. amarus* (Reichard et al., 2004a; Smith & Reichard, 2005) and is crucial to understanding the bitterling mating system. Both territorial and sneaker males frequently release sperm before a female has released eggs. Bitterling sperm has to travel from the inhalant siphon, through the mussel gill before reaching the eggs, with a delay between ejaculation and fertilisation of up to 30 seconds (Smith & Reichard, 2013). Therefore, sperm that is released just prior to egg release has a greater chance of fertilisation than sperm released after oviposition (Reichard et al., 2004a; Smith & Reichard, 2013; Smith et al., 2014a). To further improve paternity success, male bitterling perform a behaviour called ‘topping-up’ (*sensu* Parker, 1998) whereby a minimum amount of sperm is maintained

within a mussel by a guarder male through repeated pre-oviposition sperm release (Smith & Reichard, 2013; Smith et al., 2014a). Males are able to vary investment of sperm by varying ejaculate size and via repeated releases (Smith et al., 2009).

Studies by Smith & Reichard (2013) and Pateman-Jones et al. (2011) highlight interspecific differences in male sperm tactics. *R. amarus* has an approximately 6-week spawning season while *R. ocellatus* spawn for up to 6 months, with both species producing a similar number of eggs, around 150-250 (Nagata, 1985; Smith et al., 2004; Kitamura, 2005). The differing length of spawning season has affected how the males of each species invest their sperm. *R. amarus* release sperm at a higher rate, maintaining a higher baseline sperm count within a guarded mussel, and increasing ejaculation rate in the presence of a gravid female. *R. ocellatus* maintains a lower baseline of sperm within mussels, likely due to the longer spawning season making high levels of topping up more energetically demanding. Instead *R. ocellatus* increases ejaculation rate in response to rival sperm release, not female presence, suggesting direct competition with rivals is more important for this species.

Bitterling sperm and sperm production show specialised adaptations for the unusual mating system, either from selection due to sperm competition or the requirement for sperm to remain inside the mussel gill to ensure fertilisation of eggs. Notably bitterling sperm contains a large amount of mucins, which allow for a slow release of spermatozoa into the water thereby reducing the speed sperm is filtered out of the gills by a mussel (Scaggiante et al., 1999; Pateman-Jones et al., 2011). This adaptation means that *R. amarus* sperm can fertilise an egg at least 14 minutes after being released (Reichard et al., 2004a), in contrast with just 2 minutes for sperm released into freshwater without mucins (Pateman-Jones, 2007). Interspecific

comparison of the sperm of *R. amarus*, *R. ocellatus* and *R. sinensis* showed that *R. amarus* displayed higher ejaculation rates, faster swimming spermatozoa, greater mucin production and semi-cystic spermatogenesis (Pateman-Jones et al., 2011). Semi-cystic spermatogenesis involves the release of spermatids which mature asynchronously with matured spermatozoa (Manni & Rasotto, 1997). This process results in a limitation in the number of simultaneously mature gametes, but prolongs the period of effectiveness of an ejaculate. *R. sinensis* displayed higher sperm swimming speed and larger testis size than *R. ocellatus*, though these two species were otherwise similar in sperm traits, both lacking semi-cystic spermatogenesis. Species differences in sperm morphologies are likely due to different intensity of spawning rate and sperm competition in each species (Pateman-Jones et al., 2011; Smith et al., 2014a). Smith & Reichard (2013) measured sperm motility of sneakers and territorial males in *R. amarus*, but detected no difference.

1.7 SPAWNING

The bitterling spawning season is controlled by photoperiod and temperature. *A. rhombeus*, *A. tabira*, *Tanakia tanago* and *R. ocellatus* display gonadal changes to both temperature and photoperiod, often initiated by one environmental factor and arrested by the other (Shimizu & Hanyu, 1982; Ashina & Hanyu, 1983; Kawamura & Uehara, 2005; Hatakeyama & Akiyama, 2007). A similar mechanism may operate in other species. Most bitterling species spawn in spring, with a small number of autumn-spawning species, including *A. rhombeus* and *A. longipinnis* (Kawamura & Uehara, 2005; Kitamura et al., 2009). Autumn-spawning species overwinter as embryos in the mussel host for 7-9 months, undergoing diapause within the mussel (Kawamura &

Uehara, 2005). In spring-spawning species, such as *R. amarus* or *R. ocellatus*, the embryos remain within the mussel for approximately 30 days, in the case *R. amarus* (Smith et al., 2004), and between 25-26 days in *R. ocellatus* (Spence & Smith, 2013).

Table 1.1. Comparison of clutch, egg and mean ovipositor sizes for ten species and subspecies of bitterling (Shirai, 1962; Kawamura et al., 2001; Kitamura, 2005; Smith et al., 2004; Kitamura, 2006; 2007; Reichard et al., 2007a; Kitamura et al., 2009; Oshiumi & Kitamura, 2009; Kitamura et al., 2012).

Species	Clutch size	Egg length (mm)	Mean ovipositor length (mm)
R. amarus	1-6	2.4-3.1	-
R. ocellatus	4.4 ± 1.3	2.4-2.6	-
R. o. ocellatus	14	-	35
R. o. kurumeus	8.8	2-3.9	
R. atremius	16	2.8	-
Acheilognathus tabira	-	3	33
A. cynostigma	1	3.48 ± 0.19	-
A. rhombeus	-	-	28
Tanakia lanceolata	-	-	21
T. limbata	-	-	21

1.7.1 Oviposition

When a batch of eggs complete development and are ovulated, the female develops an extended ovipositor. This long, fleshy tube is used by the female to deposit eggs inside the mussel gill cavity. The ovipositor can be longer than the length of the

fish in some species, with larger females producing relatively larger ovipositors (Kitamura, 2006). Ovipositor size is highly variable, both among and within species (Table 1.1). These variations in ovipositor size have been suggested to be adaptive by permitting a female to place her eggs in regions of the mussel gill that smaller females may be unable to reach (Kitamura, 2006; Kitamura et al., 2012). Maximum length of ovipositor may be related to mechanical constraints related to forcing a clutch of eggs along the entire length of the ovipositor (Matsubara, 1994). Kitamura et al. (2012) showed that ovipositor length varies in *A. tabira* among populations depending on the size of available host mussel species. Average ovipositor length also varied over a spawning season in *R. ocellatus kurumeus*, with ovipositors longest at the peak of the spawning season and shorter at the beginning and end of the season (Kitamura, 2006). The spatial separation of clutches is theorised to be adaptive by avoiding inter-clutch competition (see Embryo Adaptations).

The mechanism of oviposition is described by Matsubara (1994). Prior to spawning females up take water, increasing bladder volume. The bladder empties directly into the ovipositor and urine, under pressure from contraction of the body and is used to force a clutch of eggs along the length of the ovipositor. To deposit eggs, a female swims forward over a mussel pressing the muscular base of the ovipositor into the exhalent siphon of the mussel. Muscles around the bladder force urine down the ovipositor, unfurling it into the mussel gill cavity. Between 1 and 16 eggs, depending on species (Table 1.1), are released and the female withdraws the ovipositor, with the whole manoeuvre taking less than 1 second. Multiple clutches of eggs can be laid in a single mussel. The eggs themselves are quite large (Table 1.1), oval shaped and dense, meaning they sink quickly. These characteristics aid the female in placing the eggs,

which lodge between the water tubes of the host mussel gills. Any eggs that fail to lodge in the mussel gill are expelled immediately by the exhalant water flow from the mussel and are quickly eaten by the female or other fish near the mussel (Smith et al., 2004).

1.8 EMBRYO ADAPTATIONS

Bitterling embryos have adaptations to withstand low oxygen environments and to mitigate the risk of dislodgement by the mussel. To increase oxygen diffusion, bitterling eggs hatch quickly (after 36 h) from the egg capsule (Kamler, 1992; Aldridge, 1999). Post-hatching the embryos develop an extensive cutaneous respiratory system with vascularised yolk sacs and fin folds for enhanced gaseous exchange (Suzuki & Hibiya, 1984). Embryos can also utilise an ethanol respiratory pathway (van Waarde et al., 1993), allowing energy production in the absence of oxygen. This pathway is also seen in goldfish (*Carassius auratus*) and crucian carp (*Carassius carassius*), which are related to bitterling and have the capacity to survive low oxygen conditions (Wissing & Zebe, 1988; van Waarde et al., 1993).

Bitterling eggs do not vary greatly in size (Table 1.1) (Smith et al., 2004), which may reflect selection to ensure they lodge within the water tubes of the host mussel. Bitterling species that are generalists in host use must have eggs adapted for a variety of mussel gill structures (Liu et al., 2006). Embryos of *Rhodeus* sp. have an unusual 'lightbulb-like' shape (Suzuki & Hibiya, 1984; Suzuki & Jeon, 1988a) while *Acheilognathus* sp. and *Tanakia* sp. appear to have more spherical eggs (Suzuki & Jeon, 1988b; 1991). Scaly tubercles, along with yolk-sac projections are present in many species (Suzuki et al., 1985; Suzuki & Hibiya, 1984; Suzuki & Jeon, 1988a; Suzuki et al., 1989) but not all (Suzuki & Jeon, 1991). These structures appear to function in

limiting the ejection of eggs and embryos from mussels. Once an embryo becomes motile, after 4 days in the case of *R. amarus* (Dmitrijeva et al., 1985), they actively swim against water flow and away from light, both adaptations to maintain their position in the host mussel.

Embryo and egg mortalities can be high during incubation. Kitamura (2005) demonstrated mortality in the spring spawning *R. ocellatus kurumeus* to be 30% during the peak of the spawning season, but 70% outside this period. Early mortalities were due to ejection, likely due to low temperatures slowing development of traits that prevent ejection. Embryos later in the season suffered suffocation as higher temperatures due to reduced oxygen availability, elevated density-dependent competition and increased oxygen consumption by both embryo and host.

1.9 OVIPOSITION DECISIONS

1.9.1 Mussel inspection

Both males and females 'inspect' mussels prior to spawning (Duyvené de Wit, 1955; Wiepkema, 1961; Smith et al., 2004). The fish align along the length of the mussel, with their snout placed 1-5 mm from the exhalant siphon of the mussel and body tilted upwards at about 75 degrees from horizontal (Heschl, 1989). A variety of cues that may play a role in oviposition choice are potentially detectable from the exhalant siphon of a mussel, including water flow velocity, oxygen content, mussel odour and embryo odour. Whether the function of mussel inspection is the same in males and females and among species is not clear (see Mussel cues below)

1.9.2 Skimming

Skimming behaviour, termed ‘touching’ by Wiepkema (1961), occurs when a female presses the base of her ovipositor to a mussel siphon but does not unfurl the ovipositor or release eggs (Smith et al., 2001; Smith & Reichard, 2005; Smith et al., 2007) and the function of this behaviour is disputed.

One possibility is that skimming behaviour signals female intention to spawn in a mussel (Smith et al., 2001). The rapid, forward movement of the female when skimming is highly visible and may serve to encourage males to participate in a spawning (Smith & Reichard, 2005; Reichard et al., 2007c; Spence et al., 2013). Skimming may therefore improve fertility assurance for the female, though potentially erodes female mate choice. Smith & Reichard (2005) did however show that female skimming rates increased in the presence of larger and more brightly coloured males. This demonstrates that females retain a measure of mate choice when males sneak.

Skimming potentially generates a three-way conflict between female, guarder and sneaker (Alonzo & Werner, 2000; Jennions & Petrie, 2000; Reichard et al., 2007c). Female behavioural changes during sneaker participation have been shown in other fishes, such as the bluegill sunfish (*Lepomis macrochirus*) (Fu et al., 2001), coho salmon (*Oncorhynchus kisutch*) (Watters, 2005), bluehead wrasse (*Thalassoma bifasciatum*) (Warner et al., 1995) and Atlantic salmon (*Salmo salar*), which also performs ‘false spawning’ (Esteve, 2007).

1.9.3 Mussel cues

The bitterling mating system relies entirely on mussels to incubate the young (Duyvené de Wit, 1955). The embryos and mussel have a close interaction over an extended period of the bitterling’s development, so the characteristics of the mussel selected for

oviposition will have a large effect on juvenile fitness, and host choice in bitterling might be expected to be under strong selection. The two primary causes of embryo mortality in bitterling are suffocation and dislodgement from the mussel (Smith et al., 2000a; 2001; Kitamura, 2005). Despite receiving a constant water flow inside the mussel gill, the embryos are in competition for oxygen, not only with the mussel but each other (Aldridge, 1999). This competition can result in slowed development and possible death (Smith et al., 2001; Kitamura, 2005; Reichard et al., 2006; Spence & Smith, 2013).

The dissolved oxygen concentration of water emerging from the exhalent siphon of a mussel, and water flow velocity has been proposed as cues used by bitterling for assessing mussel quality as a spawning site (Smith et al., 2001; Mills & Reynolds, 2002b), though these variables are potentially correlated, at least within mussel species.

Heschl (1989) performed the most comprehensive analysis to date of potential cues used by bitterling in selecting a mussel for oviposition. He utilised a basic artificial mussel and present *R. amarus* with various shapes of siphon and mussel in combination with water velocity, oxygen content and odour. Heschl (1989) suggested that females use a hierarchy of cues when evaluating mussel suitability, starting with olfactory cues, then water flow and, finally, visual cues. Unfortunately Heschl's (1989) study used inspection behaviour as a response variable. While it is now recognised that inspection is likely involved in assessment of mussel quality (Smith et al., 2001), repeated inspection may not signify mussel preference as conclusively as skimming or spawning (Smith et al., 2001).

The significance of mussel choice in the bitterling mating system is highlighted by female bitterling consistently selecting mussel quality over mate quality in making

spawning decisions (Smith et al., 2001; Agbali et al., 2012). Thus *R. amarus* consistently avoid spawning in the mussel *Anodonta cygnea* (Smith et al., 2000a; 2001), which is a superior competitor for oxygen than other mussel species (Smith et al., 2001), irrespective of mate identity (Smith et al., 2002; 2003). Oxygen extraction rate is not the only factor affecting oxygen conditions within the mussel. If multiple egg clutches are spawned in the same mussel, embryos compete for oxygen and can impact each other's development. When selecting a mussel host, a female bitterling chooses hosts containing the fewest embryos (Smith et al., 2000a; 2001). It is unclear whether females can perceive the presence of eggs and embryos in mussel gills, or whether they use an alternative cue as a proxy, such as dissolved oxygen.

In making oviposition-site decisions, female bitterling can not only influence the short-term survival of offspring, but also have longer-term impacts. The long-term effects of female oviposition decisions can act through disruption to development resulting in alternative phenotypes (Gluckman et al., 2005). Work by Agbali & Smith (2012) showed that *R. ocellatus* embryos raised at high density *in vitro* matured at an earlier age and smaller size than embryos reared at low density. Agbali & Smith (2012) suggested that the size reduction may be a cost of 'coping' with lower oxygen levels without causing additional mortality. In high density populations, where sneakers and guarders are equally successful (Reichard et al., 2004a), it may be beneficial to produce smaller offspring, which would tend to mate as sneakers when mature. However, this is only a benefit for males, as females will still experience strong size-dependent reproductive success (Reichard et al., 2009). It was, therefore, hypothesised that the findings were a 'coping' cost rather than a beneficial change in phenotype (Agbali & Smith, 2012). A skew in sex ratios was also found under different densities with a

greater proportion of males produced at lower densities (Agbali & Smith, 2012). This change in sex ratio is possible owing to the flexibility of sex determination in fish (Devlin & Nagahama, 2002; Wootton & Smith, 2015). Further studies will be needed to understand the mechanism for skews in sex ratio on offspring fitness.

The function of mussel inspection behaviour by male bitterling, despite being one of the most common behaviours seen during spawning, is not understood. Three hypotheses of function have been suggested; to assess sperm competition (Smith & Reichard, 2013), to detect egg release and, to direct females towards the mussel. What constitutes high mussel 'quality' for males is disputed, but is potentially different to that for females (Casalini et al., 2013). Males do show some mussel preferences (Smith et al., 2002) but will follow females to their preferred mussel (Casalini et al., 2013).

1.9.4 Conflict over mussel choice

Evolutionary conflicts in behaviour arises when a behaviour of an individual lowers the fitness of other members of the population (Partridge & Hurst, 1998). In the bitterling mating system, conflicts are potentially widespread. Intersexual conflict surrounds fertility assurance, with males possibly rationing sperm, while females skim to increase male investment (Smith & Reichard, 2005; Smith et al., 2007; 2009). Other conflicts include female preference for sneaker involvement in mating (Smith et al., 2009) and erosion of female mate choice by male dominance or sneaking (Casalini et al., 2009).

Conflicts over host choice may also occur in bitterling. While female bitterling appear to choose mussels on the basis of cues that affect embryo mortality (Smith et al., 2001), males appear to have different mussel preferences (Casalini et al., 2013) and avoid spawning in mussels experiencing sperm competition (Smith et al., 2002). These

different responses may result in sexual conflict over mussel choice, with females potentially using mussels in which offspring survival is compromised, or males using mussels in which they face an increased risk of sperm competition.

An additional source of potential conflict is among siblings (Mock & Parker, 1998), a feature of the reproductive biology of bitterling that has yet to be investigated. Due to guarding behaviour by males alongside rival sneaking attempts, developing embryos within a mussel will comprise of full and half siblings alongside unrelated individuals. As the number of clutches within a host increases, oxygen competition among embryos is expected to increase. It is unclear if any aspect of sibling relations affects survival within a mussel, for example spatial patterns. Clutches of eggs are placed in clusters within the mussel, making siblings each other's greatest competitors for oxygen. Further research into this critical stage of development is warranted, given the opportunity embryos have for maximising their inclusive fitness (Hamilton, 1964).

1.9.5 Interspecific Interactions

A little-studied area of bitterling ecology is interspecific interactions. This is likely due to the preponderance of research on *R. amarus*, which does not share its distribution with any other bitterling species. Kitamura (2007) studied the mussel use of four co-occurring species of bitterling in the River Harai, Japan; one autumn spawner (*A. rhombeus*) and three spring spawners (*A. tabira tabira, T. limbata* and *T. lanceolata*). *A. rhombeus* avoided overlap with other species due to its autumn spawning period, and this temporal segregation is thought to be an adaptive life history change to avoid competition (Kitamura, 2007). *A. tabira tabira* has shown dominance over the two other co-occurring species (Fukuhara et al., 1984) which was believed to

affect host choice in these two sub-ordinate species. *T. limbata* and *T. lanceolata* showed overlap in season, host choice and host location within the river. Further study of the role of interspecific competition on oviposition decisions in Asian bitterling are needed to understand its significance for oviposition-site decisions, especially for endangered species of bitterling sharing their habitat with invasive bitterling (Kawamura et al., 2001).

1.10 CONCLUSIONS

Bitterling fishes exhibit many adaptations to their unusual mode of reproduction, including mate and host choice, embryo and sperm adaptations and alternative mating tactics. Females exhibit complex mate choice involving both direct selection on male size (Smith et al., 2014b) but also through genetic compatibility (Agbali et al., 2010). This high level of mate choice is unusual for a resource-based mating system (Neff & Pitcher, 2005). Both male and female mussel choice is under strong selection with high mortality rates of eggs and embryos during incubation in mussels. Males exhibit conditional alternative mating tactics, often switching strategies within a single spawning, and utilising sophisticated sperm allocations associated with 'topping up' and, pre-oviposition ejaculation (Smith & Reichard, 2013). These remarkable adaptations make the bitterling fishes outstanding candidates for research on mating system evolution, and a range of questions remain to be addressed.

1.10.1 *Aims & Hypotheses*

The cues and consequences of oviposition choice require further attention in bitterling, as they appear to have a disproportionate effect on reproductive success (Smith et al.,

2001; Kitamura, 2005). A better understanding of these oviposition choices will allow not just a more complete knowledge of the bitterling mating system, but also allow this knowledge to be related to other systems.

a) While the current hypothesis is that females select mussels on the basis of oxygen availability (Heschl, 1988; Smith et al., 2001), this has yet to be proven by experimentally differentiating oxygen content and flow preference (Mills & Reynolds, 2002a) as the two cues can be correlated in some species (Davenport & Woolmington, 1982). It is expected that females will follow the current hypothesis.

b) The cues used by males, and the possible adaptive benefits to male oviposition decisions, are largely unknown. While males may match or copy female host preferences (Casalini et al., 2013), there is also evidence that they avoid mussels where the risk of sperm competition is elevated (Smith et al., 2002). The cues used by males in host choice will be investigated and I hypothesise a role of sperm competition in these cues.

c) From interpopulation studies, particularly co-evolutionary work (Reichard et al., 2010), there have been demonstrations that bitterling display great variability in their behaviour to adapt to different environments. An investigation of how bitterling behaviour may vary among populations will examine the degree of variability in this behaviour, and its responsiveness to demographic and environmental conditions.

d) It has hitherto been assumed that the cues used by bitterling in making oviposition decisions relate exclusively to mussels (Smith et al., 2001; Mills & Reynolds, 2002a). However, in a natural environment, the abundance, size and

sex of other bitterling potentially contribute a range of social cues that could affect oviposition-site decisions. These cues will be tested in a range of experiments and it is predicted that sperm competition will play a major role in the responses to these cues.

2.0 Chapter Two

SEX DIFFERENCES IN THE RESPONSES TO OVIPOSITION-SITE CUES BY A FISH REVEALED BY TEST WITH AN ARTIFICIAL HOST

2.1 ABSTRACT

Oviposition decisions can have important fitness consequences for offspring. Here the responses of European bitterling (*Rhodeus amarus*), a freshwater fish that spawns in the gills of living unionid mussels, to oviposition-site cues were investigated. Using an artificial mussel, the flow velocity, dissolved oxygen concentration and, odour cues of mussels presented to pairs of *R. amarus* were manipulated. Both sexes responded only if they were presented with the odour of living mussels. Female response was also contingent on a high dissolved oxygen concentration, while the strength of female response was determined by the difference between mussel and ambient dissolved oxygen concentration; females responded strongly to high relative host dissolved oxygen concentrations. Male response was dependent on the flow velocity of water emerging from the artificial mussel. These responses are potentially adaptive, with females responding to cues that indicate the quality of oviposition sites for incubation of eggs. Males responded to cues with implications for optimal sperm allocation. These findings experimentally demonstrate which host cues are used by bitterling while raising the possibility of a sexual conflict over host choice.

2.2 INTRODUCTION

2.2.1 Background

Irrespective of mating system, mothers exert primary control over their propagules, either through the size and number of offspring, provisioning of eggs and embryos and, in taxa in which female mate choice operates, the paternal contribution of genes to offspring (Mousseau & Fox, 1998). In many taxa the female is the sole or predominant care-giver (Clutton-Brock, 1991). One means by which a mother can contribute to offspring success is through her oviposition-site decisions (Mousseau & Fox, 1998; Roitberg, 1998; Refsnider & Janzen, 2010), particularly in species that oviposit on discrete patches of resource - such as parasitoids (Taylor et al., 1998), brood parasites (Soler, 2014), phytophagous insects (Mayhew, 1997), and seed beetles (Cope & Fox, 2003). Oviposition-site decisions have long been recognised as having significant evolutionary and ecological consequences (Adolph, 1920; Refsnider & Janzen, 2010).

Maternal oviposition-site decisions will affect the fitness of offspring if oviposition sites vary in quality. Quality may vary among resource types (e.g. among a range of host species), or because females 'superparasitise' a resource patch (i.e. they deposit their eggs on the same resource patch as other females). The immediate and longer-term fitness outcomes of maternal oviposition decisions have received attention (Shine & Harlow, 1996; Spence & Smith, 2013). In many taxa, however, males also play a role in oviposition decisions (Refsnider & Janzen, 2010).

The oviposition-site decisions of males and females need not correspond. In some mating systems males can influence female oviposition-site decisions through harassment (Córdoba-Aguilar, 2009), the transfer of ejaculatory substances during mating (Wolfner, 2002), and by controlling access to oviposition sites (Qvarnström &

Forsgren, 1998) - potentially resulting in sexual conflict (Spence & Smith, 2005). If oviposition-site decisions have different outcomes for the sexes and represent a possible arena for sexual conflict, a key question is whether the sexes attend to the same or different cues in making decisions.

2.2.2 Rhodeus amarus

Here the responses of male and female European bitterling (*Rhodeus amarus*) to oviposition-site cues were investigated. Oviposition decisions have been demonstrated to be important to bitterling survival and paternity (Smith et al., 2001; 2003; Kitamura, 2005; Reichard et al., 2006; Spence & Smith, 2013; **Chapter 1**). Thus oviposition-site cues are a key component of the bitterling mating system, with immediate (Smith et al., 2000a; Mills & Reynolds, 2002a) and longer-term (Agbali & Smith, 2012) consequences for fitness. The cues used by bitterling for oviposition-site decisions are disputed. *R. amarus* show a response to the rate of water flow from the exhalant siphon of a mussel (Smith et al., 2001; Mills & Reynolds, 2002b), but also to the dissolved oxygen concentration of the exhalant flow (Smith et al., 2000a; 2001). Separating the effects of these two cues is problematic because flow velocity and dissolved oxygen concentration are potentially correlated (Davenport & Woolmington, 1982). Identifying the individual effects of oxygen and flow could allow a better understanding of the priorities of bitterling when selecting hosts. Additionally, there is evidence that visual and odour cues and the presence and behaviour of other bitterling can influence oviposition choice (Heschl, 1989; Smith & Reichard, 2005). Males and females may not express the same host preferences, with males perhaps not having strong mussel preference (Casalini et al., 2013) and avoiding spawning in mussels experiencing sperm competition (Smith et al., 2002). Here the strength of response of *R. amarus* to

oviposition-site cues was experimentally investigated. Three discrete cues were examined: water flow velocity, dissolved oxygen concentration, and mussel odour, using an artificial mussel that permitted manipulation of each cue independently. The questions of whether single or multiple cues are used and whether males and females use the same or different cues was addressed.

2.3 METHODS

2.3.1 General methods

Approximately 350 *R. amarus* were collected from the River Kyjovka in the Czech Republic, a tributary of the River Morava, in the southeast of the Czech Republic at the centre of the distribution of European bitterling (Bryja et al., 2010). In addition, approximately 180 *Unio tumidus* mussels were collected from an adjacent oxbow lake (where both bitterling and mussels are abundant) prior to the start of the spawning season, in April 2015. Fish and mussels were transported to outdoor fiberglass tubs (1.3 x 1.3 m). Each tub was filled to a depth of 0.6 m with 1000 litres of water that had been left to dechlorinate for three days and furnished with a gravel substrate and artificial plants as refuges. Fish were stored in mixed-sex groups at low densities (approximately 30 fish per tub) and fed *ad libitum* three times daily with a mixture of frozen chironomid larvae and copepods. Mussels were stored separately from fish. Approximately one third of the water in tubs containing fish and mussels was changed twice weekly to maintain water quality. Given the low densities of bitterling, poor water quality was not a problem during the study. Fish and mussels in tubs were exposed to natural light and temperature variation, typical for mid-May in central Europe. Mean (±

SD) water temperature was 17.9 (± 2.5) °C, and there were approximately 15.5 h daylight hours each day over the experimental period.

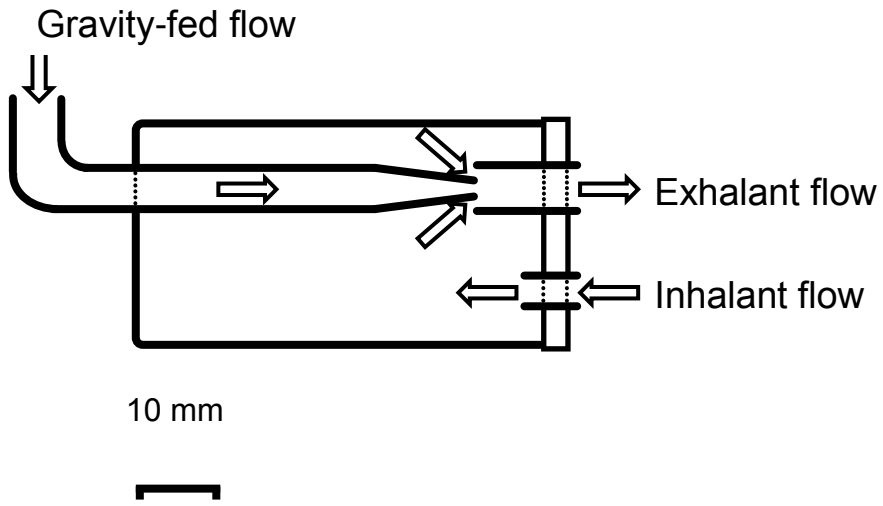
2.3.2 Experimental Design

Experiments were conducted in fiberglass tubs identical to those used to store fish and mussels. Eight experimental tubs were stocked with three male *R. amarus* and a *U. tumidus* mussel in a sand-filled plastic pot. The pot kept mussels in a fixed position while permitting them to adopt a natural orientation. Males were left for at least 24 h to settle before the start of the experiment. In each case the largest male established dominance in the experimental tubs and actively guarded the mussel. This individual served as the focal male in the experiment. Non-focal males occasionally inspected the experimental mussel when the focal male was not present, but did not participate in spawning behaviour with the focal female. While these non-focal males served to encourage guarding and territoriality by the focal male, any effects they might have had on the focal pair did not vary among experimental treatments and their presence served to make the experimental set up comparable with natural conditions.

To start the experiment, a female with an extended ovipositor (indicating a readiness to spawn), was gently caught in one of the stock tubs and transferred to a glass box measuring 220 (height) x 80 (width) x 80 (depth) mm with a mesh top to permit water exchange. The box containing the female was placed in a pre-selected experimental tub 300 mm from the mussel guarded by the focal male. Once the focal male began courtship and the female showed a response by attempting to follow him, the live *U. tumidus* was replaced with an artificial mussel and the female was released from the glass box. Any odour from the live mussel was diluted quickly within the 1000 litre experimental tub and so would not have affected behaviour towards the artificial

mussel. This experimental design was intended to accommodate the mating system of European bitterling. In nature, males are highly territorial around a patch of mussels, while females display no site attachment and range among male territories, feeding and spawning over an extensive area (Smith et al., 2004). Thus the design used, with males confined to a territory and gravid females gently introduced to these territories for short intervals, mirrored natural conditions.

Figure 2.1. Cross-sectional assembly of artificial mussels used in the study. Arrows indicate direction of water flow.



2.3.3 Artificial Mussel Design

Artificial mussels comprised a 35 mm plastic film canister measuring 50 (length) x 30 (diameter) mm with a snap on lid. The lid of the canister had two openings; an exhalant aperture of 10 mm and an inhalant aperture of 5 mm. The female bitterling releases eggs through the exhalant siphon while the male releases sperm over the inhalant siphon. Water flowed into the base of the artificial mussel under gravity,

through a constriction to elevate flow velocity, and out of the exhalant aperture (Figure 2.1). A Venturi system generated the exhalant and inhalant flow in the mussel. The Venturi effect describes the reduction in fluid pressure as a liquid is passed through a narrowed pipe. As water flows through the constricted pipe in Fig. 2.1, the water flow increases, causing a reduction in fluid pressure in the cavity of the mussel according to the Venture effect. This reduction in fluid pressure draws water into the artificial mussel through the inhalant aperture, creating both an inflow and an outflow, replicating a mussel. This design of artificial mussel permitted the source and rate of flow to be experimentally manipulated. In pilot studies males guarded artificial mussels, led females to them to spawn and ejaculated over the inhalant aperture. Similarly, females inspected the exhalant aperture of artificial mussels and spawned in them. These pilot studies demonstrate that a perfect visual replication of a mussel is not required to elicit spawning responses from bitterling, though visual cues may have a role in identifying mussels from a distance (Heschl, 1989). No negative effects of potential endocrine-disrupting chemicals derived from artificial mussels were detected. Even if present, the dilution of these chemicals in experimental tubs, combined with the extremely short time to which fish were exposed to them, meant that the reproductive system of experimental fish was unlikely to have been compromised.

2.3.4 Treatment Conditions

R. amarus were exposed to all combinations of three experimental mussel treatments; high and low flow rate, high and low dissolved oxygen concentration, and the presence and absence of mussel odour (Table 2.1) - thereby generating eight treatment combinations. Treatment combinations were imposed in a predetermined

random pattern and a total of 80 experimental trials were conducted over the study, with 10 replicates of each treatment combination.

Table 2.1. Experimental artificial mussel treatment combinations used in the study.

Mussel odour	Dissolved oxygen	Flow velocity
Present (80 mussels m^{-3})	High (7.5 mg l^{-1})	High (300 ml min^{-1})
		Low (100 ml min^{-1})
	Low (1.5 mg l^{-1})	High
		Low
Absent (0 mussels m^{-3})	High	High
		Low
	Low	High
		Low

Artificial mussels were connected by 5 mm diameter PVC tubing to a 1000 litre reservoir ('source tub') that was raised approximately 0.6 m above the level of the experimental tub in which observations were carried out. To create a high dissolved oxygen concentration (DO), water in the reservoir was strongly aerated with an air pump. To create a low dissolved oxygen concentration, nitrogen was bubbled through water in the reservoir. Dissolved oxygen concentration was monitored with a dissolved oxygen meter (HORIBA U-222). Mean ± 95% CI dissolved oxygen concentration in high oxygen treatment reservoirs was 7.48 ± 0.21 mg O_2 l^{-1}, and low treatment 1.48 ± 0.14 mg O_2 l^{-1} (Table 2.1). Algal growth in experimental tubs resulted in elevated

dissolved oxygen concentrations through photosynthesis compared to source tubs, which were free of algae. The outcome was a higher ambient dissolved oxygen level in experimental tubs than in the water emerging from artificial mussel siphons, even in the high dissolved oxygen treatment (mean ± 95% CI high dissolved oxygen treatment 10.24 ± 0.28 mg O_2 l^{-1}, low treatment 9.87 ± 0.25 mg O_2 l^{-1}). Under natural conditions mussels consume between 7% and 90% (Smith et al., 2001) of oxygen flowing over their gills, depending on species, gravidity and parasitism by bitterling (Smith et al., 2000a; 2001; Reichard et al., 2007a). Therefore, the dissolved oxygen concentration of water emerging from the siphons of artificial mussels, which declined to between 73% and 15% of the concentration of the surrounding water, accurately reflected the range naturally encountered by bitterling. To accommodate this feature of the study in the analysis, the difference in the ambient dissolved oxygen concentration in experimental tubs and the source tub supplying water to the artificial mussel was calculated and used as an additional covariate (see below). The mean ± 95% CI difference in dissolved oxygen concentration in the high oxygen treatment was 2.77 ± 0.26 mg O_2 l^{-1}, and low treatment 8.40 ± 0.24 mg O_2 l^{-1}. The volume of water flowing into the experimental tubs from the artificial mussels during observations (a maximum of 3 litre) was too low to have a measurable impact on oxygen conditions inside the experimental tubs (1000 litre).

High and low flow rates were obtained by clamping the tube connecting the artificial mussel to the water source until the desired flow rate was achieved. The presence of mussel odour was achieved by placing 80 *U. tumidus* mussels in the 1000 litre source reservoir, a procedure previously used to elicit oviposition behaviour in *R. amarus* (Heschl, 1989). Water quality was maintained with twice weekly water changes

of approximately 250 litre of water and mussels were fed daily with phytoplankton. Mussels filter water at a rate of about 2 litres hour^{-1} (Smith et al., 2001), hence the entire contents of the source reservoir would pass across the gills of the stocked mussels several times in 24 h. The source tubs were stocked with mussels two weeks prior to the start of the experiment to ensure a maximum concentration of odour was reached and pilot trials showed that bitterling were responsive to this concentration of odour. Any mussel odour cues in the small quantities of water transferred to experimental tubs when fish were moved (approximately 1 litre) would be rapidly diluted.

2.3.5 Behavioural Observations

After replacing the live mussel with an artificial mussel the behaviour of the female and focal male was observed for 10 minute or until a spawning occurred. Behaviours recorded were, for the male: inspection of the exhalant aperture and ejaculation over the inhalant aperture (see **Chapter 1** for full description). In females a record was made of inspection of the exhalant aperture and skimming, whereby the female sweeps quickly over the exhalant aperture, which she touches with the base of her ovipositor but without inserting her ovipositor into the mussel or releasing any eggs. Skimming behaviour encourages males to release sperm, and may function in assuring fertilisation of eggs (Smith & Reichard, 2005). Skimming has been proposed as a proxy for female mussel preference (Wiepkema, 1961; Candolin & Reynolds, 2001). Only one spawning occurred during observations, possibly due to the imperfect replication of a living mussel with an artificial one. Consequently, oviposition was not a suitable response variable for analysis, and skimming behaviour was instead used as a measure of female mussel preference (Heschl, 1989; Mills & Reynolds, 2002b). The use of skimming and sperm release as measures of mussel preference are a key improvement

on previous artificial mussel studies (Heschl, 1989; Mills & Reynolds, 2002b). This is possible due to the more realistic mussel design eliciting a high levels of preference behaviour (crucially behaviours other than inspections) as a measure of preference. These two studies also disagreed on the most important cue to bitterling; Heschl (1989) suggested oxygen, while Mills & Reynolds (2002b) suggested flow rate.

After completion of observations the dissolved oxygen concentration and temperature of the experimental tub were measured and the female and focal male were captured and measured for standard length (from the tip of the snout to the end of the caudal peduncle). Individual fish were not used again in the study. After completion of the study all fish and mussels were returned to the sites from which they were originally collected. A total of eight artificial mussels were used in the study. Individual artificial mussels were randomised among treatments.

2.3.6 Statistical analysis

Prior to applying statistical models, a data exploration was carried out (Ieno & Zuur, 2015). Homogeneity and zero inflation in the response variable were examined and collinearity between explanatory variables was investigated using variation inflation factors. Outliers in the data were identified visually using Cleveland plots. Male mussel inspection behaviour was found to be collinear with ejaculation frequency. Male inspection behaviour was subsequently dropped from the analysis, since sperm release over a mussel was taken to indicate an investment in a particular mussel and to better represent male mussel preference. Similarly, female mussel inspection behaviour, which was collinear with skimming, was dropped from the analysis. Linear models were fitted to data for male response (ejaculation frequency) and female response (skimming

frequency). These linear models fit a best-fit a line through the data, using multiple covariates. Because males and females could potentially influence the oviposition preferences of the opposite sex, the response variables of the opposite sex were included, along with experimentally manipulated mussel cues, as covariates when fitting the models. Male and female response variables were not collinear.

The data contained a high incidence of zero counts (50% for ejaculation frequency, 80% for skimming behaviour), though with responses distributed equitably among treatment combinations. Consequently, zero-altered (hurdle) models with Poisson (ZAP) or negative binomial (ZANB) distributions were employed (Zuur et al., 2009) using the *pscl* package ver. 1.4.6 (Jackman, 2014) in the R statistical environment, ver. 3.3.2 (R Development Core Team, 2017).

Zero-altered, or "hurdle" linear models are partitioned into two parts, with a binary process modelling zeros and positive counts, and a second process modelling only positive counts using a zero-truncated model (Hilbe, 2014). The binomial part of the model first demonstrates how the covariates affect the binomial chance of a behaviour occurring. Subsequently, when the behaviour has occurred, the poisson model describes how the covariates affect the how much of a behavioural response is seen, i.e. the repeated behaviours. This modelling approach enabled us to separately identify the mussel cues that elicited the occurrence of a behaviour (binary part), and the frequency of that behaviour when it occurred (zero-truncated part). For males a ZANB model was fitted as:

$$Ejaculation_i \sim ZANB(\mu_i, \pi_i, k)$$

$$E(Ejaculation_i) = \frac{1-\pi_i}{1-P_0} \times \mu_i \text{ where } P_0 = (\frac{k}{\mu_i + k})^k$$

$$var(Ejaculation_i) = \frac{1-\pi_i}{1-P_0} \times (\mu_i + {\mu_i}^2 + \frac{{\mu_i}^2}{k}) - (\frac{1-\pi_i}{1-P_0} \times \mu_i)^2$$

$$log(\mu_i) = flow_i + muss_i + oxy_i + oxdiff_i + msl_i + fsl_i + temp_i + tub_i + skim_i$$

$$logit(\pi_i) = flow_i + muss_i + oxy_i + oxdiff_i + msl_i + fsl_i + temp_i + tub_i + skim_i$$

Where $Ejaculation_i$ is the number of ejaculations by focal males in observation i assuming a negative binomial distribution with mean μ, probability π and dispersion k (Zuur et al., 2009). The variables $flow_i$, $muss_i$ and oxy_i are categorical covariates with two levels corresponding with artificial mussel water flow, mussel odour and dissolved oxygen, respectively. The variables $oxdiff_i$, msl_i, fsl_i, and $temp_i$ are continuous covariates corresponding with difference in dissolved oxygen concentration between artificial mussel and experimental tub (mg l^{-1}), male standard length (mm), female standard length (mm) and water temperature of experimental tub (°C) respectively. The variable tub_i was included to control for an effect of experimental tub and $skim_i$ was a continuous covariate that corresponded with female skimming frequency and was included to accommodate the effect of female behaviour on male mussel preferences. This Zero Inflated Negative Binomial (ZANB) model, models both the occurrence and frequency of ejaculation behaviour by European bitterling in this experiment, with the covariates of artificial flow, odour, dissolved oxygen, dissolved oxygen difference, temperature, experiment location (tub), the number of female skimming behaviours and the size of male and female. For females a ZAP model was fitted as:

$$skim_i \sim ZAP(\mu_i, \pi_i)$$

$$E(skim_i) = \frac{1-\pi_i}{1-e^{-\mu_i}} \times \mu_i$$

$$var(skim_i) = \frac{1-\pi_i}{1-e^{-\mu_i}} \times (\mu_i + {\mu_i}^2) - (\frac{1-\pi_i}{1-e^{-\mu_i}} \times \mu_i)^2$$

$$log(\mu_i) = flow_i + muss_i + oxy_i + oxdiff_i + msl_i + fsl_i + temp_i + tub_i + ejac_i$$

$$logit(\pi_i) = flow_i + muss_i + oxy_i + oxdiff_i + msl_i + fsl_i + temp_i + tub_i + ejac_i$$

Where $skim_i$ is the number of skims by focal females in observation i assuming a Poisson distribution with mean μ and probability π (Zuur et al., 2009). The covariate $ejac_i$ was included to accommodate the effect of male behaviour on female mussel preferences. This Zero Inflated Poisson (ZAP) model, models both the frequency of skimming behaviour by European bitterling in this experiment, with the covariates of artificial flow, odour, dissolved oxygen, dissolved oxygen difference, temperature, experiment location (tub), the number of male ejaculations and the size of male and female.

Best-fit zero-altered models were selected based on second-order Akaike's Information Criterion (AICc; Akaike, 1973) using the *AICcmodavg* package ver. 2.1-0 (Mazerolle, 2016) by removing predictor variables from the full models until the model with the lowest AICc values were identified. To assess the robustness of each model 1000 datasets were simulated from the best-fitting models and compared these with observed data, using the procedure of Zuur & Ieno (2016) for hurdle models.

2.4 RESULTS

The presence of mussel odour cues was essential for determining whether males responded to a mussel with ejaculations (Figure 2.2, Table 2.2). In the presence of odour cues, the frequency of ejaculation was positively associated with high water flow velocity but negatively with male size (Figure 2.2, Table 2.2). Similarly, the presence of both mussel odour cues and a high dissolved oxygen concentration was needed for eliciting female skimming behaviour over artificial mussels (Figure 2.3, Table 2.2). In the presence of these cues, the frequency of skimming by females was negatively

related to the magnitude of the difference between ambient dissolved oxygen in experimental tubs and that emerging from the artificial mussel siphon (Figure 2.3, Table 2.2). There was also a negative association between female skimming frequency and focal male size (Figure 2.3, Table 2.2). Simulated data generated from the best-fit models generated distributions that complied with observed data.

Table 2.2. Results of best-fit zero-altered negative binomial (ZANB)[a] and zero-altered Poisson (ZAP)[b] models testing the effects of mussel parameters on the responses of male and female *R. amarus*. Bold text indicates $P < 0.05$.

		Occurrence model				Frequency model			
Sex	Parameter	γ	SE	Z	P	β	SE	Z	P
Male[a]	Intercept	-1.34	2.99	-0.45	0.654	5.77	2.16	2.68	0.008
	$Flow_{(high)}$	-	-	-	-	0.83	0.42	1.99	**0.047**
	$Mussel_{(present)}$	1.99	0.52	3.84	**<0.001**	-	-	-	-
	$DO_{(high)}$	0.91	0.52	1.76	0.079	0.24	0.42	0.56	0.577
	Male size	-0.01	0.07	-0.04	0.971	-0.11	0.05	-2.15	**0.031**
	Skimming	-	-	-	-	-0.15	0.11	-1.32	0.187
Female[b]	Intercept	-1.95	3.94	-0.50	0.620	-2.97	60.03	-0.05	0.961
	$Flow_{(high)}$	-	-	-	-	-0.14	0.35	-0.40	0.688
	$Mussel_{(present)}$	3.28	1.09	3.02	**0.003**	9.89	60.00	0.16	0.869
	$DO_{(high)}$	1.50	0.70	2.15	**0.032**	-	-	-	-
	O_2 difference	-	-	-	-	0.19	0.07	2.62	**0.009**
	Male size	-0.06	0.09	-0.70	0.481	-0.17	0.06	-2.57	**0.010**

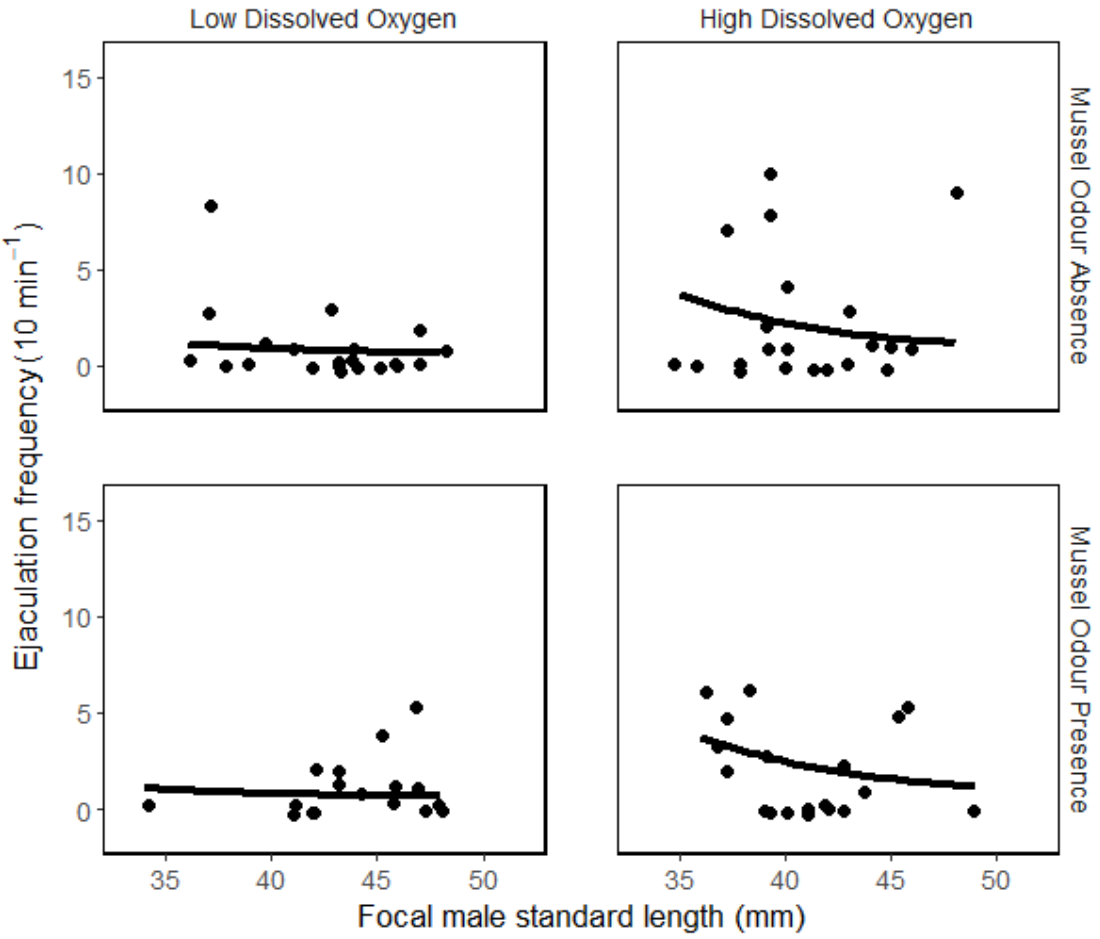


Figure 2.2. Ejaculation frequency (per 10 minutes) by focal male *R. amarus* over an artificial mussel with and without mussel odour cues and low and high flow velocity against focal male standard length (mm) modelled using a zero-altered negative binomial (ZANB) model. Black circles are observed data. Jitter used to illustrate overlapping points.

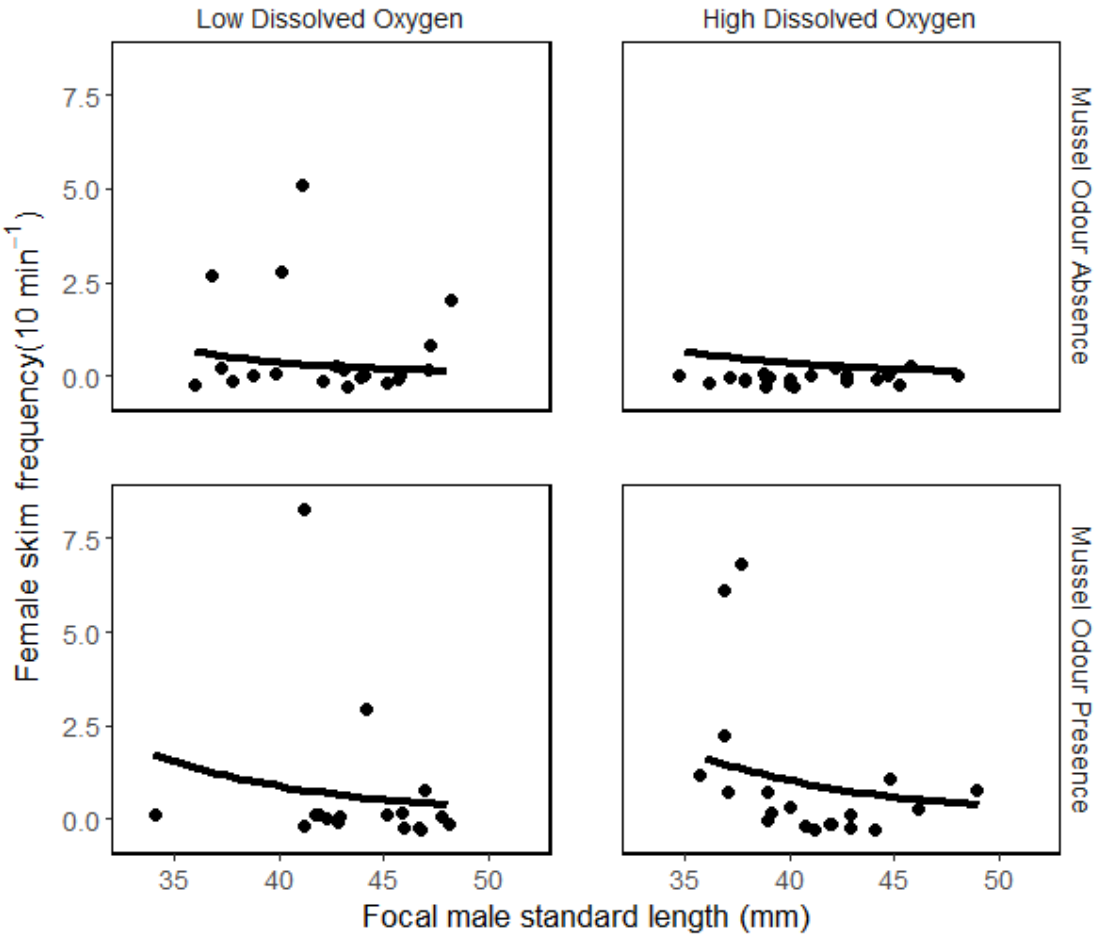


Figure 2.3. Skimming frequency (per 10 minutes) by focal females over an artificial mussel with and without mussel odour cues and low and high dissolved oxygen concentrations in the exhalant flow against focal male standard length (mm) modelled using a zero-altered Poisson (ZAP) model. Black circles are observed data. Jitter used to illustrate overlapping points.

2.5 DISCUSSION

The aim of this study was to identify the cues used by *R. amarus* in responding to oviposition sites. Appropriate responses to host cues are a key component of the mating system of this species (Smith et al., 2004), as well as other taxa (Refsnider & Janzen, 2010). Zero-altered statistical models were fitted to discriminate which cues were

responsible for the occurrence of a response to a cue, and (when a response did occur) its magnitude (Zuur et al., 2009; Hilbe, 2014).

2.5.1 Odour Cues

Both sexes expressed a positive response to water conditioned with the odour of living mussels; without this cue the reaction to artificial mussels was negligible (Figure 2.2, 2.3, Table 2.2). This response ensures that time and energy are only invested in living mussels, not water flows originating from some other source. Additional information may also be obtained from mussel odour cues. While *R. amarus* are generalists, potentially using a range of mussel species for oviposition, other bitterling species are specialists, using just one or two (Liu et al., 2006; Kitamura et al., 2012). In these cases, as bitterling appear not to attend to visual cues that discriminate mussel species (Mills & Reynolds, 2002b), species-specific odour cues may play a role in mussel choice (Reichard et al., 2007a). Chemosensory cues are crucial for the oviposition-site decisions in other taxa, including *Drosophila* spp. (Riffell, 2013), fig wasps (Hossaert-McKey et al., 1994), mosquitos (Afify & Galizia, 2015) and parasitoids (Godfray, 1994). In *D. melanogaster*, research on the mechanistic basis to oviposition-site decisions has demonstrated a role for specific volatile compounds that activate specific neurons expressing a specific odorant receptor; thus a single dedicated olfactory pathway determines oviposition choice in this species (Dweck et al., 2013). An understanding of the mechanistic basis of a response to mussel odour by bitterling may provide insights into interspecific variation in host specialism in these fishes and artificial mussel is an ideal tool to achieve this goal.

2.5.2 Dissolved Oxygen Cues

Females showed a significant response to a high dissolved oxygen concentration (Figure 2.3, Table 2.2). Oxygen availability is critical to egg and embryo development and survival during incubation in the mussel gill. Bitterling eggs are relatively large compared to other similarly sized fish, allowing them to fit in the interlamellar spaces of a mussel gill but consequently have a high *per capita* oxygen requirement (Aldridge, 1999). Given that mussels sometimes host well over 100 bitterling eggs (Smith et al., 2001; Kitamura, 2005), competition for oxygen inside the mussel gill can be severe, both among embryos and between embryos and host, and it is notable that embryo mortality rates in mussels are strongly density dependent (Smith et al., 2000a; 2001; Agbali & Smith, 2012; Spence & Smith, 2013), presumably due to asphyxiation (Aldridge, 1999; Kitamura, 2006). Consequently, natural selection is predicted to favour a preference for cues that indicate directly whether a mussel is hosting the eggs and embryos of other females, or indirectly through the decline in quality of a mussel as a result of superparasitism. Thus the response by females for mussels with high concentrations of dissolved oxygen in the exhalant flow of the artificial mussel appears adaptive, indicating to a female a mussel in good condition that contains few other embryos. Such avoidance of superparasitism is particularly well understood in parasitoids (Godfray, 1994; Gandon et al., 2006) where in some wasps, females make oviposition decisions associated with interspecific, intraspecific and self-superparasitism via 'patch marking', chemical cues left by females during oviposition (van der Hoeven & Hemerick, 1990; Viser, 1993; Harvey, 2000). Whether female bitterling can detect bitterling eggs and embryos in mussel gills is not currently known, however the indirect detection of superparasitism from dissolved oxygen levels in the mussel exhalent flow likely operates in the bitterling system.

While female *R. amarus* showed a significant threshold response to high dissolved oxygen conditions, the strength of female response to artificial mussels was predicted by the difference between the dissolved oxygen concentration of the mussel exhalant flow and the ambient oxygen concentration; the lower the exhalant flow dissolved oxygen concentration relative to ambient, the lower the female response (Table 2.2). The implication of this finding is that the female response to an oxygen cue is labile and based on comparative evaluation, rather than a fixed response to a threshold dissolved oxygen concentration. This outcome fits in the context of the ecology of bitterling and their mating system. The spawning season of *R. amarus* is relatively protracted, starting in mid-April and typically ending in mid-June (Konečná & Reichard, 2011). As the spawning season advances, mussels fill with eggs and embryos and progressively decline in quality as incubation sites (Kitamura, 2005; Smith, 2017). Water temperatures also progressively increases, with a concomitant decline in dissolved oxygen concentration. The capacity to distinguish the relative, rather than absolute, quality of an individual mussel is, therefore, critical in accommodating this temporal pattern in oviposition-site quality based on dissolved oxygen conditions inside the mussel.

Dissolved oxygen availability plays a key role in the oviposition decisions of a number of other fish species (Wootton & Smith, 2015). For example, in beaugregory damselfish (*Stegastes leucostictus*) the rate of development and survival of embryos are dependent on oxygen availability, and spawning sites are selected on this basis, with dissolved oxygen as a cue (Payne et al., 2002). In salmonids, females assess substrate quality and hyporheic flow prior to preparing spawning redds and oviposition

(Chapman, 1988; Bernier-Bourgault & Magnan, 2002; Brabrand et al., 2002; Esteve, 2005).

2.5.3 Water Velocity Cues

Male *R. amarus* responded to water flow velocity from artificial mussels with an elevated ejaculation frequency. The approach taken in this study does not allow the framing of this preference as 'choice' by the males. Males did not choose exclusively to release sperm in mussels displaying higher flow – they released more sperm in response to increased flow from the mussel (Figure 2.2). This positive, plastic response by males to water flow may reflect an unusual aspect of the bitterling mating system. Male *R. amarus* perform multiple ejaculations over mussels, even without a female present, ejaculating over a guarded mussel on >200 occasions daily, under natural conditions (Smith et al., 2009). This pattern of sperm release appears to function in maintaining a baseline level of spermatozoa in a mussel's gills (Smith & Reichard, 2013), thereby increasing fertilization chances should a female oviposit in the mussel. Sperm released into a mussel potentially undergoes passive loss from its gills as it filters water. The rate that males 'top-up' mussels with sperm differs between bitterling species, and is sensitive to the presence of rivals and females in spawning condition (Smith et al., 2014a). Filtration rates vary naturally among (Smith et al., 2001) and within host mussel species (Mills & Reynolds, 2002b). Smith & Reichard (2013) speculated that because mussels filter water at different rates (either due to species or individual differences) males might be sensitive to mussel flow rate and should respond to elevated flow rates by increasing ejaculation rates to keep mussels topped-up with sperm (*sensu* Parker, 1998). The results of the present study support this hypothesis (Table 2.2, Figure 2.2). Thus while variation in mussel flow rates did not inhibit male host preference, the

results demonstrate that males are capable of adjusting their behaviour adaptively to their current host. Male response to flow may evaluate mussel suitability for offspring survival, however flow has been positively correlated with oxygen consumption and ejections of embryos from European mussels (Mills & Reynolds 2002b, Smith et al. 2001).

2.5.4 Male Size

Male size was negatively associated with the frequency of ejaculation and also female response to mussels (Table 2.2). Male size determines dominance in bitterling (Smith et al., 2003; Casalini et al., 2009), with the largest males tending to act as guarders and smaller males acting as sneaks (Smith et al., 2004). This pattern is a common feature of the mating systems of fishes (Wootton & Smith, 2015) and other taxa (Arnqvist & Rowe, 2005). Smaller male bitterling have relatively (though not absolutely) larger testis size (Smith et al., 2014b) and typically compete with rivals through sperm competition rather than direct aggressive contests (Reichard et al., 2004a), which may explain the higher ejaculation rate of smaller males in the present study. The reason for a greater female response to smaller males is unclear. Male size and dominance do not appear to play a role in female mate choice, though large dominant males are typically able to monopolise mussels and thereby to achieve high reproductive success (Reichard et al., 2007c; 2009; Casalini et al., 2009). Male nuptial colour similarly has not been demonstrated to have a direct effect on female mate choice (Reichard et al., 2005; Casalini et al., 2009). Without measuring further male traits such as genetic compatibility, it is not possible to account for this apparent elevated response by females to smaller males.

2.5.5 Conclusion

Overall the results demonstrated that males and females responded to common, but also contrasting mussel cues. Both sexes responded almost exclusively to artificial mussels with the odour of living mussels, but while males failed to respond to dissolved oxygen levels, females showed a response to a high dissolved oxygen concentration and large relative difference in oxygen concentration between the artificial mussel and ambient. In contrast, while females did not respond to differences in water flow from the artificial mussel, males responded to higher flows by elevating their ejaculation rate (Table 2.2, Figure 2.2). These differences may reflect different adaptive priorities for males and females. Thus, while females attend to cues that reflect mussel quality as a site for incubation of young stages (Smith et al., 2001; 2002; Agbali et al., 2010; Agbali & Smith, 2012), males instead appear sensitive to the risk of sperm competition (Spence et al., 2013), and select mussels based on different measures of quality (Smith et al., 2002; 2003; 2014b; Casalini et al., 2013). A sexual conflict over responses to oviposition sites in *R. amarus* may, consequently, arise. Sexual conflict occurs when the evolutionary interests of individuals of the two sexes diverge (Parker, 1979), with a potential to generate sexually antagonistic selection (Lessells, 2006). In the context of the bitterling mating system, responses to oviposition-site cues are a potential arena for sexual conflict, with females maximizing offspring fitness through attending to the dissolved oxygen concentration of water emerging from the mussel exhalant siphon, and males maximizing fertilization success through sperm competition by responding to water flow velocity and the behaviour of rivals. These differences appear to manifest themselves as overt behavioural conflicts between spawning partners (Smith et al., 2002). Over the course of a spawning event males repeatedly attempt to lead females

away from mussels with nearby rivals while females frequently select alternative mussels on the basis of offspring survival. I am not aware of any other mating system with conflicting responses to oviposition-site cues like those seen in *R. amarus*.

3.0 Chapter Three

RESPONSE TO SPERM CUES DURING OVIPOSITION BY *R. amarus*

3.1 ABSTRACT

Oviposition-site decisions by females can have significant fitness consequences for offspring. In the case of externally-fertilising species, a key function of selecting a site for oviposition may be in ensuring fertilisation success. Males also have a role in selecting oviposition sites, though the choices of males and females may not always correspond. This study tested whether European bitterling (*Rhodeus amarus*), a small freshwater fish that fertilises and incubates eggs within freshwater mussels, were responsive to the presence of sperm in making oviposition-site decisions. Female bitterling potentially experience sperm limitation, such that their eggs go unfertilised. Sperm stripped from males was added to the inhalant siphons of mussels during courtship. Males and females did not respond to the experimental addition of sperm, but were more likely to respond to behaviours performed by the other sex (skimming or sperm release). These results suggest that female bitterling make adaptive decisions to ensure the fertility of their eggs on the basis of visual, but not olfactory, cues that indicate the likelihood that their eggs will be fertilised. Male bitterling also utilised visual cues to evaluate spawning likelihood. These visual cues are the behaviour of the other sex during pre-spawning behaviour. These findings demonstrate sophisticated behavioural responses by females to ensure fertility.

3.2 INTRODUCTION

3.2.1 Sperm as a Resource

Sperm is a vital resource of mating systems, important in both fertility assurance and sperm competition. External fertilisers may experience dilution of sperm and compound issues such as incompatibility and insufficient sperm (Eberhard, 1996). This 'sperm limitation' (insufficient sperm for all eggs to be fertilised) incurs high infertility costs upon females (Warner et al., 1995; Eberhard, 1996). Sperm depletion (males being unable to release sufficient sperm) may result from increased expenditure of ejaculates due to sperm competition with rival males or intentional conservation of sperm for future sperm competition and has serious consequences for male paternity success (Dewsbury, 1982; Birkhead & Fletcher, 1995; Warner et al., 1995; Parker, 1998). There are costs of sperm limitation across a wide range of taxa. Female bluehead wrasse (*Thalassoma bifasciatum*) (Warner et al., 1995), lekking sandflies (*Lutzomyia longipalpis*) (Jones, 2001) and Soay sheep (*Ovis aries*) (Preston et al., 2005) have all been demonstrated to suffer reduced fertility from sperm limitation. In these examples, females mated with dominant males who were either sperm depleted or who reduced the size of their sperm releases to conserve sperm. Other common causes of sperm limitation are sperm quantity (Wedell et al., 2002) and compatibility (Nakatsura & Kramer, 1982), both of which are likely to occur when mating with a single dominant male. Females can mate multiply to increase access to sperm and reduce incompatibility (Jennions & Petrie, 2000), as seen in red flour beetles (*Tribolium castaneum*) (Pai et al., 2005). Females encountering such costs are predicted to evolve the capacity to gauge sperm presence to better evaluate their risk of sperm limitation.

3.2.2 Knowledge of Sperm Investment

For males, response to the risk of sperm competition was modelled by Ball & Parker (1998). They showed that with increased accuracy of knowledge of the sperm competition risk, males invested more in a previously mated females. This effect has been shown experimentally in a mealworm beetle (*Tenebrio molitor*) (Gage & Baker, 1991) and medflies (*Ceratitis capitate*) (Gage, 1991) where the presence of rival males (a proxy for sperm competition risk) resulted in a male releasing up to twice as much sperm. The effect of female knowledge of sperm investment is less well understood, despite such an evaluation being potentially adaptive. The present study will investigate how the act of sperm investment is identified, and how this may affect a mating system.

3.2.3 Rhodeus amarus

The effects of sperm as an oviposition cue will be investigated in the European bitterling (*Rhodeus amarus*) model system (**Chapter 1**). The experimental control afforded by manipulation of the mussel oviposition site makes the bitterling system an excellent model species for research on sperm competition and limitation. Bitterling females experience sperm limitation when only a single male participates in spawning (Smith & Reichard, 2005; Smith et al., 2009; **Chapter 1**). In response, females may perform a skimming behaviour to prompt a resident male, and any nearby non-resident sneakers, to release more sperm over a mussel and thereby improve fertility assurance for the female (Smith & Reichard, 2005; Smith et al., 2007). Male bitterling can ejaculate 21 times an hour during the spawning season and experience sperm limitation over multiple spawnings (Pateman-Jones, 2007; Smith et al., 2009). Host availability affects sperm competition and egg release rates across bitterling populations (Casalini, 2013). In populations of low fish density relative to mussels, more spawnings are pair spawnings and the risk of sperm limitation and depletion is greater. It would therefore

be expected that any response to sperm limitation would be stronger in populations with low fish density relative to mussels.

In the present study, bitterling response to sperm odour cues were tested by adding sperm or a water control to a mussel inhalant siphon and recording differences in female oviposition-site decisions and frequency of male sperm releases. It was predicted that both male and female bitterling would display increased behavioural responses to the addition of rival sperm.

3.3 METHODS

3.3.1 Study populations

Three populations of *R. amarus* were chosen for this experiment. Two study populations were located in Turkey, one from Lake Sapanca (40° 42′ N, 30° 15′ E) and the other from the River Ballica (40° 00′ N, 29° 25′ E), both in western Anatolia. A further population was located in the Czech Republic, in the River Kyjovka (48° 47′ N, 17° 01′ E), a tributary of the River Dyje (Danube basin). These three populations are genetically distinct, reflecting differences in their time of divergence (Bryja et al., 2010).

The abundance of bitterling and mussels in each population differed, with the result that there were differences in fish densities relative to mussel densities among the three populations. While few studies have examined the temporal stability of mussel populations, they appear stable over a time scale of at least 20 years (Smith, 2017), but possibly longer. Here the term “mean crowding” (Lloyd, 1967) is used to describe this relative densities, with high mean crowding indicating a high density of fish to mussels. Mean crowding (Lloyd, 1967) is a more meaningful measure than density or resource

availability alone, since it encapsulates a measure of absolute density and the degree of competition among individuals for a resource. Casalini (2013) quantified mean crowding in these three populations by placing a set of mussels at each site and recording the number of eggs laid. Casalini's (2013) study showed that the mean number of eggs deposited in mussels in the River Ballica (4.69 ± 0.80 mussel^{-1}) was significantly higher than in Lake Sapanca (0.18 ± 0.10) and the River Kyjovka (0.31 ± 0.11). Male bitterling from the River Ballica were also more brightly coloured and aggressive than the other two populations, which corresponded with the hypothesis that male-male competition was greater in the Ballica population (Casalini, 2013). This finding implies that mean crowding in the River Ballica was greater than at the other two sites, with a greater risk of sperm competition and probability of oviposition, an inference supported by direct observation in nature (Phillips, pers. obs.).

Turkish *R. amarus* were collected using baited fish traps. The R. Kyjovka *R. amarus* were collected using an electroshocker designed for use with small fish. We used a specially designed battery-driven pulse DC apparatus (Lena, Bednář Olomouc, Czech Republic), with a small diameter anode that selectively targeted fish smaller than 100 mm. Electrofishing was considered the least stressful method of capture (Janáč 2009), with much lower impacts on non-target stream biota than Seine netting, which involves indiscriminate capture and abrasive damage to fish. All fish were transported to the Institute of Vertebrate Biology, Brno, Czech Republic and stored in aquaria measuring 1200 (length) x 400 (width) x 400 (depth) mm, with a sand substrate, filters and aquatic plants as refuges. All fish were fed a mixture of frozen chironomid larvae and commercial fish granules. Fish were exposed to a natural light:dark cycle of 15.5:8.5 h. *Unio pictorum* mussels were used as spawning sites in all experimental

treatments. This is a locally common mussel species and is found at all three study sites. Mussels were collected by hand from an oxbow lake adjacent to the R. Kyjovka before the start of the bitterling spawning season. *U. pictorum* is regularly utilised by *R. amarus* for oviposition (Smith et al., 2004; Reichard et al., 2010). Collected mussels were stored in an outdoor pool and fed with phytoplankton.

3.2.2 Experimental Design

For experimental treatments, sperm was stripped from two males from the same population as the tested male. The fish were placed in a Petri dish and gentle pressure was applied across the abdomen to release sperm. These males were returned to their home aquarium and not used again in the study. Their condition was monitored following stripping and survival was 100%. Aquarium water was added to the petri dish to make up 2 ml of sperm solution which was used within 30 minutes of stripping. A sperm count of 10 random samples was conducted utilising a haemocytometer viewed under a microscope. Mean (± SD) sperm counts were 6.0×10^6 (± 2.6) ml^{-1}. A control water solution was prepared by briefly placing two males in a Petri dish to which 2 ml of aquarium water was added but without stripping sperm to control for any effect of olfactory cues derived from handling males during stripping.

3.3.3 Experimental Protocol

Two males and a *U. pictorum* in a sand-filled container were placed in an experimental aquarium measuring 500 (length) x 400 (height) x 300 mm (depth) and allowed to settle for 1 hour. The larger male became territorial and was designated as the focal male. The rival male rarely participated in mating and did not release sperm over the mussel as they were quickly chased away by the focal male. Thus, this rival male served to

stimulate territorial behaviour by the focal male, but played no role in the experiment itself. A randomly selected female with an extended ovipositor from the same population as the focal male was gently removed from a stock tank and confined in a 500 ml flat-sided glass container with a mesh top to allow water exchange. After being allowed to settle, the female was released into the aquarium and the behaviour of the female and focal male were recorded for 12 minutes. The behaviours recorded were: focal male and female inspection of the mussel exhalant siphon, focal male sperm release and female skimming behaviour. If spawning occurred the experiment was stopped and time of oviposition recorded; after spawning, male behaviour changes abruptly and includes aggression towards the female and elevated frequency of ejaculation (Smith et al., 2004).

Fish were randomly assigned to either a sperm solution or a water control for their first observation, followed by the corresponding treatment in the second observation. Control or sperm solutions were added to the experimental aquarium at intervals of 0, 4 and 8 minutes. 0.6 ml of the treatment solution was added, at each interval, to the inhalant siphon of the mussel using a pipette. Once focused on spawning, bitterling are not readily distracted and trials showed the application of the treatment did not disrupt their behaviour. After completion of observations, the standard length of the focal male and female fish were measured to the nearest 1 mm. Mussel total length and water temperature were also recorded. Due to a limited numbers of gravid females in all test populations, some were re-used, but their identity was recorded to control for potential pseudo-replication in the analysis. The same protocol was followed for all three populations. A total of 54 pairwise replicates were collected, with 18 pairs for each population using 54 individual males and 40 gravid females.

3.3.4 Statistical Analysis

Data exploration was carried out according to Ieno & Zuur (2015). Data were examined for outliers in the response and explanatory variables. No outliers were identified by dotchart. Homogeneity and zero inflation were examined in the response variables and nature of relationships between response and explanatory variables were plotted to fully understand the data. Continuous coefficients were standardised to facilitate model convergence. Male and female inspection behaviour were identified as collinear, based on estimated variance inflation factor, and were subsequently dropped from the analysis. For males, ejaculation over a mussel was used as the response variable, since this represents a measure of male investment in a mussel or particular oviposition. Due to over dispersion of Poisson models and the inclusion of female identity as a random effect, male sperm release was analysed using a binomial GLMM (Generalised Linear Mixed Model). The GLMM applies a link function (such as a log link) to a linear model to fit a line, described by the covariates, to the data. The "mixed" aspect of the model, applies a different line to each random variable (female identity in this case) and collates the lines to better describe the data.

$$Ejaculation_{ij} \sim Binomial(\pi_{ij}, N_{ij})$$

$$E(Ejaculation_{ij}) \sim N_{ij} \times \pi_{ij}$$

$$\eta_{ij} = fTreat_{ij} + fPop_{ij} + msl_{ij} + fsl_{ij} + mtl_{ij} + temp_{ij} + skim\ rate_{ij} + female_j$$

$$\text{logit}\ (\pi_{ij}) = \eta_{ij}$$

$$female_j \sim N(0, \sigma^2_{female})$$

$Ejaculation_{ij}$ is the probability of ejaculation by a male with focal female *j*, which was assumed to follow a binomial distribution with mean π_{ij} and variance π_{ij} x $(1-\pi_{ij})$, with a

logit link function. The logit function ensures the fitted probability of ejaculation falls between 0 and 1. $fTreat_{ij}$ is a categorical covariate with two levels, corresponding with experimental treatment; sperm or control solution. $fPop_{ij}$ is a categorical covariate with three levels, corresponding with the three study populations; Kyjovka, Sapanca, Ballica. The model also contained linear effects for male standard length (msl_{ij}), female standard length (fsl_{ij}), mussel total length (mtl_{ij}), water temperature ($temp_{ij}$), and rate of female skimming behaviour ($skim\ rate i_j$). The random intercept $female_j$ was included to introduce a correlation structure between observations with the same female. The GLMM models the probability of a male European bitterling releasing sperm in this experiment, according to the treatment applied, their population, the temperature, the size of the male, the female and the mussel, and the skimming rate of the female.

For females, oviposition represents a definitive measure of investment by females in a spawning site and was chosen as the response variable. Female oviposition data were modelled using a binomial GLMM as:

$$Ovi_{ij} \sim Binomial(\pi_{ij}, N_{ij})$$

$$E(Ovi_{ij}) \sim N_{ij} \times \pi_{ij}$$

$$\eta_{ij} = fTreat_{ij} + fPop_{ij} + msl_{ij} + fsl_{ij} + mtl_{ij} + temp_{ij} + ejac\ rate_{ij} + female_j$$

$$\text{logit}(\pi_{ij}) = \eta_{ij}$$

$$female_j \sim N(0, \sigma^2_{female})$$

Ovi_{ij} is the probability of egg release by female j. In addition to variables included in the GLMM for male response, the model also contained linear effects for male ejaculation rate ($ejac\ rate_{ij}$). The GLMM models the probability of a female European bitterling ovipositing an egg in this experiment, according to the treatment

applied, their population, the temperature, the size of the male, the female and the mussel, and the ejaculation rate of the male.

3.4 RESULTS

The rate of female skimming was a significant predictor of the probability of male sperm release (Table 3.1, Figure 3.1). However, there were no statistically significant effects of experimental treatment, male, female or mussel size, or water temperature. There was no statistically significant effect of experimental treatment on female probability of oviposition (Table 3.2). However, there was a positive effect of male sperm release rate on probability of oviposition (Table 3.2, Figure 3.2).

Table 3.1. Summary of Bernoulli GLMM to predict the probability of male ejaculation during trials. Individual females were fitted as random intercepts. Bold text indicates $P < 0.05$.

Model parameter	Estimate	s.e.	*z*	*P*
Fixed intercept$_{(Ballica)}$	2.91	0.77	3.77	**<0.001**
fTreat$_{(control)}$	-0.257	0.6	-0.428	0.669
fPop$_{(Kyjovka)}$	-1.08	0.856	-1.258	0.208
fPop$_{(Sapanca)}$	-0.327	0.844	-0.387	0.7
msl	0.314	0.326	0.965	0.335
fsl	-0.245	0.379	-0.646	0.518
mtl	0.388	0.298	1.29	0.194
temp	0.0748	0.304	0.246	0.806
skim rate	1.47	0.595	2.46	**0.014**

Table 3.2. Summary of Bernoulli GLMM to predict the probability of female oviposition. Individual females were fitted as random intercepts. Bold text indicates $P < 0.05$.

Model parameter	Estimate	s.e.	*z*	*P*
Fixed intercept$_{(Ballica)}$	-0.0988	0.489	-0.202	0.84
fTreat$_{(control)}$	-0.613	0.427	-1.43	0.151
fPop$_{(Kyjovka)}$	-0.0367	0.63	-0.058	0.954
fPop$_{(Sapanca)}$	-0.3	0.682	-0.439	0.661
msl	0.0838	0.222	0.378	0.705
fsl	0.0975	0.278	0.351	0.725
mtl	-0.241	0.222	-1.08	0.279
temp	-0.25	0.219	-1.14	0.253
ejac rate	0.724	0.228	3.18	**0.001**

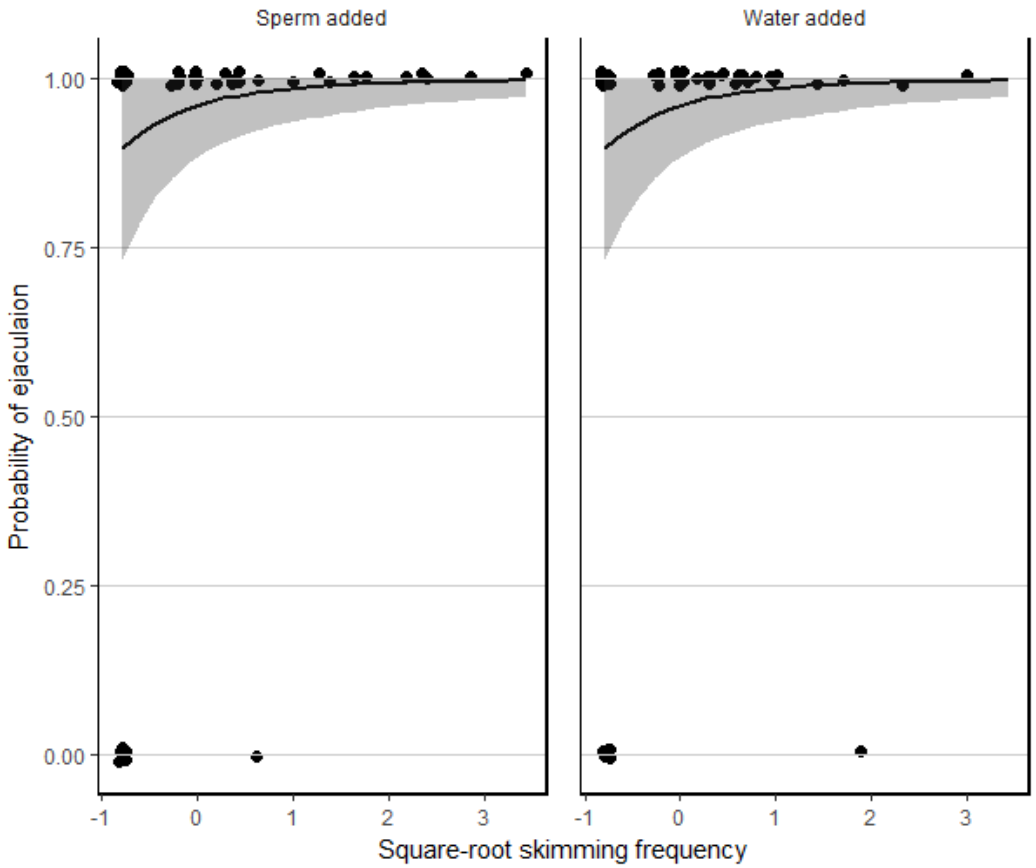


Figure 3.1. Mean fitted frequency (solid line) of probability of ejaculation by European bitterling (*Rhodeus amarus*) and 95% confidence intervals (shaded area) against the square root of female skimming frequency for a Bernoulli GLMM. The males were exposed to two experimental treatments (sperm stripped from another male or a water control). Black circles are observed data with jitter added to clarify points.

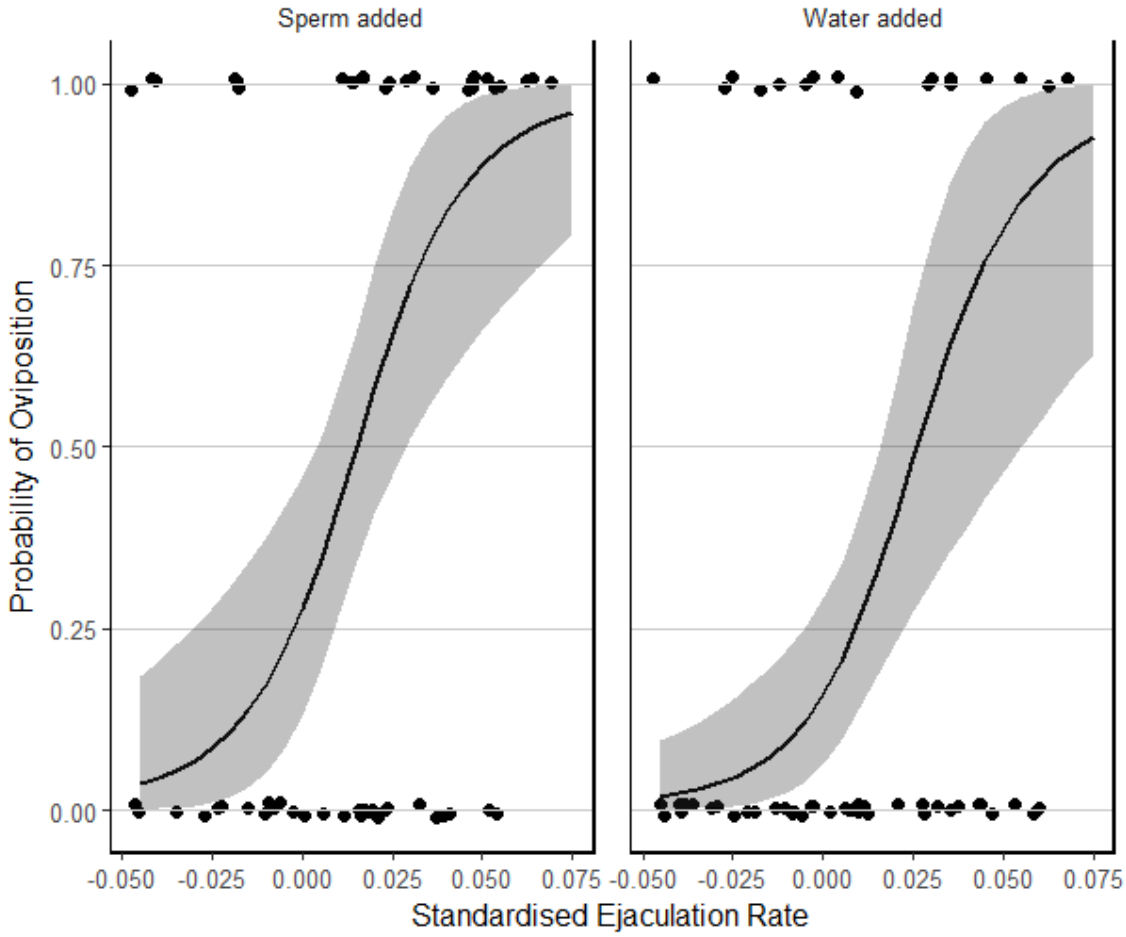


Figure 3.2. Mean fitted frequency (solid line) of probability of oviposition by female European bitterling (*Rhodeus amarus*) and 95% confidence intervals (shaded area) against standardised focal male sperm release frequency for a Bernoulli GLMM. The females were exposed to two experimental treatments (sperm stripped from a male or a water control). Black circles are observed data with jitter added to clarify points.

3.5 DISCUSSION

The aim of this study was to identify whether sperm odour cues influenced bitterling oviposition cues. No effect of sperm treatment or population on the probability of female oviposition or male sperm release was found (Table 3.1, 3.2, Figure 3.1, 3.2). However, the behaviour of the other sex did influence egg laying and sperm release

(Table 3.1, 3.2, Figure 3.1, 3.2). These findings do not support the hypothesis that bitterling directly detect sperm to inform investment of resources in hosts, but suggest that the visual cues of ejaculation and skimming behaviour play a role in oviposition decisions.

3.5.1 Male Response

A challenge in using bitterling for research has been identifying the function of male mussel inspection behaviour (Smith et al., 2003; Phillips et al., 2017). Previous work has clarified three hypotheses for the role of male inspection: to assess sperm competition (Smith & Reichard, 2013), to detect egg releases (Phillips et al., 2017), or to signal the presence of a mussel to females (Smith et al., 2003). The results directly relate to the function of inspection. During inspection males may detect the olfactory cues of rival male sperm (Smith & Reichard, 2013) and Smith et al., (2002) demonstrated that guarder males increased their ejaculate expenditure in response to rival males releasing sperm a mussel. Smith et al., (2003) artificially added thawed, non-motile sperm to mussels under natural conditions and found no effect on guarder male sperm release. The results from the present and previous studies suggest that males respond to rival male sperm releases under natural and aquarium conditions (Smith et al., 2002; Table 3.1), but do not respond to fresh or frozen sperm alone (Smith et al., 2003; Table 3.1). These findings suggest that males measure the intensity of sperm competition not by olfactory cues but by visual ones. Sperm release is a conspicuous behaviour that can be recognised by territorial males, allowing them to evaluate rival sperm investment visually. The only variable in the model that had a significant effect on sperm release was female skimming behaviour (Table 3.1). This result supports previous studies on skimming behaviour, which is known to elicit sperm release by

males (Smith & Reichard, 2005). Information on rival sperm release will help a male allocate sperm adaptively to invest more in spawnings in which they have a good probability of fertilising some eggs (Ball & Parker, 1998; Smith & Reichard, 2013). These findings also help clarify the function of male inspection function by ruling out sperm detection, and instead implicating detection of egg release (Phillips et al., 2017) or as a signal of mussel presence to a female (Smith et al., 2003).

3.5.2 Female Response

Female European bitterling respond to the costs of sperm limitation through a variety of behaviours. Females are more likely to release eggs in the presence of sneakers (Smith et al., 2002; 2003; 2009) and, in the related rose bitterling (*R. ocellatus*), show a preference for genetically compatible males (Agbali et al., 2010; Reichard et al., 2012). Female bitterling also utilise 'skimming' behaviour to encourage greater investment from guarder males and sneakers (Smith & Reichard, 2005). The present study identifies that they additionally evaluate the frequency of sperm release when making oviposition decisions but do so using visual, not olfactory cues (Table 3.2). Quantifying male investment in a spawning can allow females to assess whether their eggs will be fertilised and may also serve as a cue to indicate the likelihood that multiple males participate in a mating.

The present study also expands our understanding of female oviposition cue detection. **Chapter 2** identified that females use oxygen cues to select mussels, thereby maximising offspring survival. The present results suggest that females also utilise visual social cues to make oviposition decisions to maximise fertilisation success, and possibly also to enhance genetic variability or compatibility in the offspring (Table 3.2). The bitterling mating system utilises other visual cues in host detection (Heschl, 1989)

and male competition (Casalini et al., 2013) demonstrating that visual cues are reliable function in decision making. Male ejaculatory behaviour ostensibly appears a reliable as signal of sperm release, however there has been a suggestion that males occasionally 'fake' sperm releases to encourage spawning by females while economising on sperm (Pateman-Jones, 2007). Further research on this potential intersexual conflict is needed.

A possible caveat to this study is that experimentally released sperm may not have been released in the correct location or at an appropriate concentration. Through trials, the placement of sperm was practiced and dyed sperm was visibly seen entering the mussel inhalant siphon and exiting the exhalent siphon. In pilot studies, a total of 10 random sperm count samples were taken of the stripped sperm treatment solution with mean (± S.D.) spermatozoa concentrations found to be 6×10^6 ($\pm 2.6 \times 10^6$) ml^3. Smith et al. (2009) recorded male ejaculate size under natural conditions at around 8×10^4 ml^3. Thus, the concentration of spermatozoa in the sperm treatment was approximately two orders of magnitude greater than an ordinary ejaculation. If bitterling are able to detect the olfactory cues from a natural ejaculate, this amount of sperm would be sufficient to elicit a response.

3.5.3 Detection of Sperm

Sperm limitation and depletion have fitness consequences for both sexes and are seen in many species (Møller, 1991; Levitan 1993). Response to the intensity of sperm competition is also predicted to change male behaviour (Ball & Parker, 1998). Despite this, there are few examples of males or females detecting sperm investment, through olfactory or visual cues. This absence of published studies suggests either physiological limitations to sperm detection or a lack of research on the subject. The present study suggests that the visual detection of sperm release behaviours is an appropriate proxy

for detecting sperm releases. Further sperm response experiments, such as the present study, should be conducted on species experiencing high levels of sperm competition or sperm limitation. Coho salmon (*Oncorhynchus kisutch*) are another species that responds to sperm limitation with behavioural adaptations. Female Coho salmon display preference for sneaker 'jack' salmon, spending more time digging nests and releasing more eggs than with 'hooknose' guarders (Watters, 2005). Female bluehead wrasse (*Thalassoma bifasciatum*) display a similar response to sneakers (Fu et al., 2001). In these cases it is unclear whether females are evaluating fertility assurance or consistently prefer the presence of a sneaker. Such species would be prime candidates for testing sperm evaluation by females. In lekking sandflies (Jones, 2001) and Soay sheep (Preston et al., 2005), both of which experience costs of sperm limitation, it may also be expected that sperm detection behaviours could evolve.

3.5.4 Conclusions

Through accurate visual evaluation of repeated male sperm releases, female European bitterling are able to improve their reproductive success by adaptive allocation of gametes during oviposition. The attention to host choice cues relating to offspring survival is commonplace (Phillips et al., 2017; **Chapter 2**). However, the behavioural adaptations to sperm limitation displayed by female *R. amarus* are extensive, and beyond what has been demonstrated in other taxa. In medflies and mealworm beetles only the presence of a rival male elicited males to release more sperm (Gage & Baker, 1991; Gage, 1991). Bitterling demonstrate a more precise evaluation of sperm competition by identifying rival investment visually. Previous research has shown that male bitterling increase investment in a mating linearly with a single rival, male while decreasing their investment as multiple rivals engage in sperm competition

(Smith et al., 2002; Smith & Reichard, 2013). The present results suggest a means of quantifying sperm competition intensity by male bitterling during mating. Further understanding of the mechanisms behind this complex decision making would further our understanding of sperm competition and the contribution of males to oviposition decisions.

4.0 Chapter Four

SPATIAL OVIPOSITION DECISION BY THE EUROPEAN BITTERLING

4.1 ABSTRACT

Morphological ‘handedness’, an asymmetry in form which may affect behaviour, has been demonstrated in fish and humans and is maintained by frequency dependent selection. Bitterling are a freshwater fish that incubate their eggs within one of four equally sized gills of freshwater mussels. Bitterling embryos experience strong density dependent selection and it has been suggested that bitterling females may have some measure of gill choice when placing eggs. The occurrence of ‘oviposition handedness’ was investigated in the European bitterling (*Rhodeus amarus*). Embryo placement in mussel gills was monitored in a pairwise experiment (controlling for effect of individual mussel morphology) across three populations that varied in mean ‘crowding’. No individual effect of left or right gill choice was found across any population, though a significant preference for inner gills was found. This study demonstrated that individual female bitterling place eggs randomly with respect to mussel bilateral symmetry and display no ‘handedness’. More eggs were oviposited into the inner gills. This may be adaptive in avoiding the developing mussel embryos (glochidia) that are incubated in the outer gills of female mussels. It remains to be demonstrated whether the avoidance of outer gills is an active choice by the female or a result of changes to flow patterns within the mussel due to glochidia blocking water tubes.

4.2 INTRODUCTION

The precise location of an embryo within an oviposition patch or host can affect their subsequent success, depending on the degree of variation in microhabitat (Orr & Resh, 1992). Examples of microhabitat level oviposition-site decisions include superparasitism by parasitoid wasps, which can lead to frequency-dependent selection in the host (van Alphen & Visser, 1990). Also, in the cowpea seed beetle (*Callosobruchus maculatus*), females allocated more eggs to larger seeds when presented with multiple seeds, maximising the resources available per offspring (Cope & Fox, 2003). These and other studies demonstrate the significance of small-scale spatial decisions during oviposition and the consequences for offspring development (Parker & Courtney, 1984; Nishimura, 1993). Frequent dependent selection has been associated with morphological and behavioural laterality (Bisazza et al., 1998). Behavioural laterality has been studied in predation and aggression in teleost fishes (Takeuchi & Hori, 2008; Takeuchi et al., 2010). However behavioural laterality has yet to be investigated in oviposition.

4.2.1 Rhodeus amarus

Bitterling are small freshwater fish that spawn eggs in freshwater mussels. It has been proposed that bitterling fish may experience frequency-dependent selection for oviposition decisions with the result that they place their eggs in different regions of the mussel gill to avoid clutches being placed close to one another and experiencing negative density-dependent effects. In the Japanese rose bitterling, *Rhodeus ocellatus kurumeus*, ovipositor length, was observed to vary over the course of a spawning season, which Kitamura (2006) proposed as a mechanism that limited density-dependent competition among embryos. In addition to depth of placement, egg location could potentially vary between the left and right gills of a mussel, if females express a

form of ‘oviposition handedness’, whereby females may have a tendency to consistently place each clutch of eggs the spawn in either the left or right gill (Figure 4.1). As an adaptation, this spawning tendency might be favoured if it ensured egg clutches were consistently placed in the gill of a mussel without risk of ejection. If this was the case, the prediction would be that left and right ‘handedness’ female oviposition morphs would be maintained in equal abundance through frequency-dependent selection (Cain & Sheppard, 1954).

4.2.2 Morphological handedness

Two examples of handedness morphs in fish are the scale-eating cichlid (*Perissodus eccentricus*) and the bucktooth tetra (*Exodon paradoxus*) (Hori, 1993; Van Dooren et al., 2010; Hata et al., 2011). These fish feed by approaching another fish from behind, on the left or right side, and removing scales using their specialised jaws. Individuals have a ‘jaw flank specialisation’ and consistently approach their prey from one side. Asymmetry in their jaw development mean that they are more efficient at removing scales from one side. If one jaw morph increases in abundance, prey species become more vigilant on one flank, reducing the success of that morph. Hence frequency dependent selection maintains left and right-jawed morphs at approximately the same frequency (Hata et al., 2011).

Similarly, the maintenance of left-handedness in humans has been proposed as arising through frequency-dependent selection. Left-handers represent a minority in all human populations (Raymond & Pontier, 2004) and this trait, which has a heritable component, is associated with a number of fitness costs (Coren & Halpern, 1991). Thus, the maintenance of left-handedness in the face of these costs represents an enigma. A proposal is that left-handedness confers a frequency-dependent advantage in fights, with

the advantage arising because left-handers are relatively rare. An outcome is that right-handers are unaccustomed to confronting left-handed rivals, whereas left-handers routinely confront right-handers (Raymond et al., 1996). Studies of a variety of sporting contests, as well as violent human societies, support this hypothesis (Raymond et al., 1996; Faurie & Raymond, 2005).

Here, oviposition 'handedness' is investigated in the European bitterling (*Rhodeus amarus*). The aim of the study was to identify whether individual bitterling express handedness in the distribution of eggs during oviposition (consistently preferring one gill of a mussel over the other). It was further investigated if there was intraspecific variation across three bitterling populations which naturally vary in density – with the prediction that fish from populations with higher levels of mean crowding (*sensu* Lloyd, 1967), would experience stronger frequency dependent selection and, consequently, higher levels of handedness.

4.3 METHODS

4.3.1 Study populations

Rhodeus. amarus were collected for this study from Lake Sapanca and River Ballica in Turkey and the River Kyjovka in the Czech Republic. River Ballica experiences a markedly lower host density than the other two populations (Casalini, 2013). Mussel populations appear stable of at least 20 years (Smith, 2017) so it is likely that the difference in host density in Ballica and Sapanca has existed for many generations of bitterling. Turkish *R. amarus* were collected using baited fish traps and transported to the Institute of Vertebrate Biology in the Czech Republic. River Kyjovka fish were collected using in May 2016 using electrofishing kit designed for use with small fish.

We used a specially designed battery-driven pulse DC apparatus (Lena, Bednář Olomouc, Czech Republic), with a small diameter anode that selectively targeted fish smaller than 100 mm. Fish were transported to aquaria measuring 1200 (length) x 400 (width) x 400 (depth) mm with sand substrate and fed a mixture of frozen *Chironomidae* larvae and commercial fish granules. The natural light:dark cycle was 15.5:8.5h.

Unio pictorum mussels were used as hosts for the experiment. This is a common mussel species throughout the distribution of *R. amarus* and is abundant at the three sites from which experimental fish were collected and is regularly utilised by *R. amarus* for oviposition (Smith et al., 2004; Reichard et al., 2010). Mussels were collected prior to the start of the spawning season from an oxbow lake adjacent to the River Kyjovka and checked for the presence of bitterling embryos. Mussels were stored prior to use in an outdoor pool and fed with phytoplankton.

4.3.2 Experimental Protocol

Clutch placement within a mussel host was tested in three populations of *R. amarus*. A female with an extended ovipositor (indicating readiness to spawn) was placed in an experimental tank measuring 500 (length) x 400 (height) x 300mm (depth) already containing a *Unio pictorum* in a sand filled container and a male from the same population. Mussels were checked for eggs every hour by gently opening the shell using a mussel opening device while the mussel was submerged. If eggs were found, their number and location was recorded (Figure 4.1). If no eggs were found, mussels were returned to the experimental tank for an additional two hours, with two further checks for eggs each hour. The protocol had to control for any effect of individual mussels on

which gill embryos were placed in. A female was allowed to spawn in two mussels in two separate trials, egg location recorded, and then the same mussels were then presented to a second female following an identical protocol. This procedure allowed us to measure consistent handedness effects of females while controlling for mussel effects. After completion of trials, the female was measured for standard length and returned to a stock tank and were not used again. The total length of the mussel was also recorded to the nearest 1mm. The same protocol was used for all three populations with a total of 25 fully crossed replicates of 50 females and mussels.

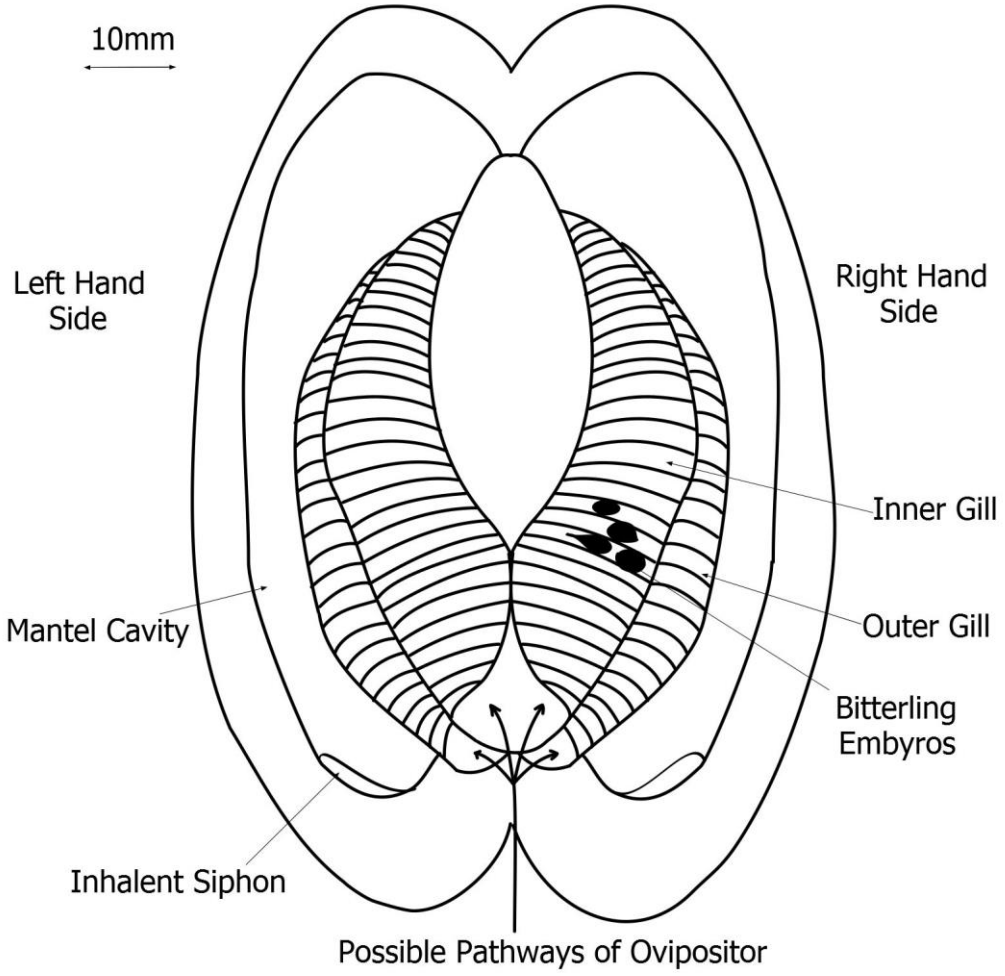


Figure 4.1. Illustration of *U. pictorum* mussel gills. The route of the female ovipositor during spawning is indicated by arrows. The outer gill is displayed under the inner gill and are of equal size.

4.3.3 Statistical analysis

Egg position was analysed as presence/absence in either the left or right gills of the mussel or the inner or outer gills. A data exploration was carried out according to Ieno & Zuur (2015). The data were found to be highly zero inflated (72% zeros) and consequently the data were modelled using a Bernoulli GLMM with replicate as a random effect. The experimental design allowed two females to lay eggs in the same two mussels, allowing control over the effects of individual female preference but also any effect of individual mussels on egg position. Given the fully crossed design, female oviposition handedness was examined using the interaction of egg location (left or right) x female. The interaction of egg location x mussel was used to identify if mussels consistently biased the location of clutches. The GLM (Generalised Linear Model) took the form:

$$Egg\ Release_{ij} \sim Binomial(\pi_{ij})$$

$$E(Egg\ Release_{ij}) = \pi_{ij}$$

$$\text{var}(Egg\ Release_{ij}) = \pi_{ij} \text{ x } (1 - \pi_{ij})$$

$$\text{logit}\ (\pi_{ij}) = \eta_{ij}$$

$$\eta_{ij} = fHand_{ij} * fFemaleID_{ij} + fHand_{ij} * fMusselID_{ij} + fGill_{ij} + fPop_{ij} + fsl_{ij} + mtl_{ij} + replicate_{j}$$

$$replicate_{j} \sim N(0,\ \sigma^2_{\text{replicate}})$$

$Egg\ Release_{ij}$ is the probability of egg release for female i assuming a Bernoulli distribution with mean π_i and variance π_i x (1 - π_i). The variable f$Hand_{ij}$ is a categorical covariate with two levels; left or right. $fFemaleID_{ij}$ is a categorical variable with two levels; 1st female or 2nd female to spawn. $fMusselID_{ij}$ is a categorical variable with two levels; 1st mussel or 2nd mussel to be spawned in. $fGill_{ij}$ is a categorical covariate with

two levels; inner or outer. fPop_{ij} is a categorical covariate with three levels, corresponding with the three study populations; Kyjovka, Sapanca, Ballica. $fsl.std_{ij}$ is the standardised continuous variable of female standard length. $mtl.std_{ij}$ is the standardised continuous variable of female standard length. This GLM models the probability of a female European bitterling releasing an egg in this experiment, according to the interaction of gill side with female ID and mussel ID, along with gill inner or outer as a factor, population of the bitterling and the size of the female and mussel. All the data from this study will be made available in the Dryad Digital Repository.

4.4 RESULTS

Females deposited eggs consistently more in the inner gills of mussels (Table 4.1, Figure 4.2). There was no evidence of oviposition handedness by female bitterling, or of a tendency for mussels to bias egg location. There was no population effect on egg location, and no effect of either female size or mussel size (Table 4.1).

Table 4.1. Summary statistics of Bernoulli GLMM to predict egg position in the gills of freshwater mussels. Fully crossed replicates were fitted as random intercepts with standard deviation of 0. Bold text indicates P < 0.05.

Model parameter	Estimate	s.e.	*z*	*P*
Intercept	-0.07	0.30	-0.21	0.830
Hand$_{(right)}$	0.02	0.41	0.06	0.956
FemaleID$_{(2nd)}$	-0.35	0.35	-0.99	0.324
MusselID$_{(2nd)}$	0.00	0.34	0.01	0.991
Gill$_{(outer)}$	-1.79	0.26	-6.84	**<0.001**
fPop$_{(Kyjovka)}$	-0.35	0.31	-1.13	0.261
fPop$_{(Sapanca)}$	-0.23	0.32	-0.73	0.465
fsl	0.17	0.13	1.32	0.187
mtl	0.06	0.12	0.46	0.646
Hand x *FemaleID*	0.40	0.48	0.84	0.402
Hand x *MusselID*	-0.40	0.48	-0.83	0.405

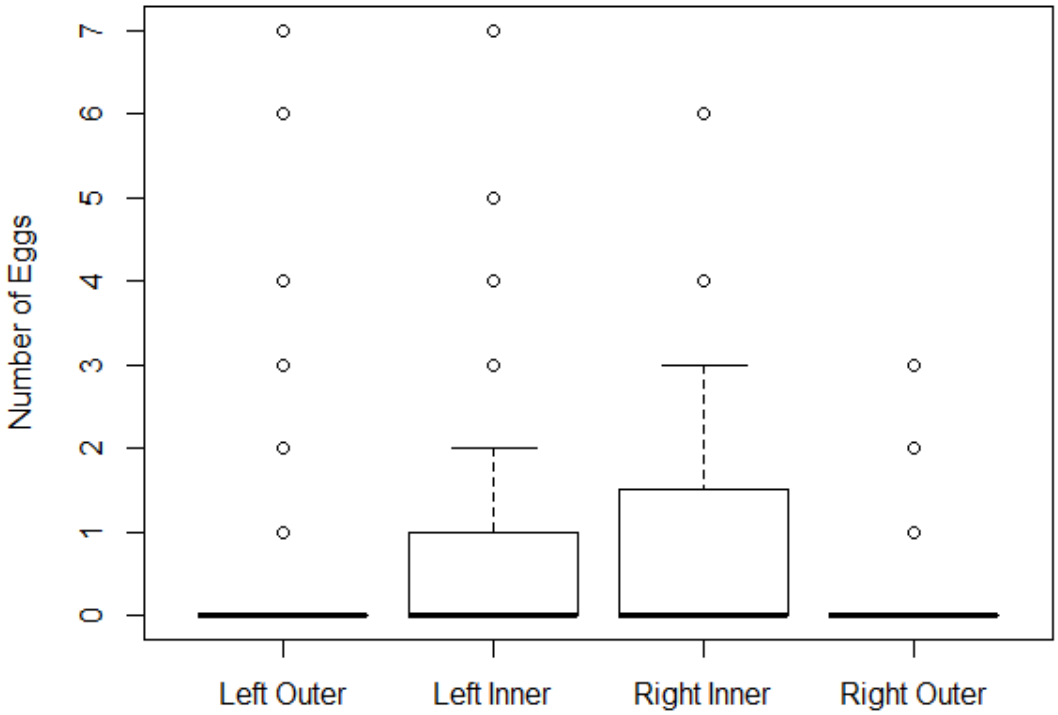


Figure 4.2. Egg placement by female European bitterling (*Rhodeus amarus*) in the gills of the common host *Unio pictorum*.

4.5 DISCUSSION

The aim of the study was to investigate oviposition handedness in *R. amarus* in the gills of its host mussel during oviposition. No evidence of handedness effects in either female bitterling or mussels was detected. However, there was a highly significant effect of egg placement demonstrated by the study, with a pronounced tendency for females to deposit their eggs in the inner gills, rather than the outer gills, of a mussel (Table 4.1, Figure 4.2).

4.5.1 Gill Choice

Unionid mussels have larval stages (termed glochidia) that are obligate parasites of freshwater fishes. Developing glochidia are incubated in the outer gills of female and hermaphrodite individuals, which are modified for this purpose, and termed marsupium. Incubating glochidia have a high oxygen requirement (Tankersley & Dimock, 1993) and can obstruct bitterling eggs from successfully lodging in the water tubes of the mussel (Aldridge, 1999; Kitamura, 2005). Work by Kitamura (2006) showed that *R. ocellatus kurumeus* avoided oviposition in the outer gills of female mussels, though they distributed their embryos evenly across inner and outer gills in male mussels. This pattern was attributed to the incubation of glochidia. While the present study did not record mussel sex, the results lend further support to the discrimination of inner and outer gills, possibly due to the effects of incubating glochidia. Interestingly, Kitamura (2006) found female inner gill selection only occurred during pair spawning (one male and one female). In group spawning events (multiple males and females), the inner gills were utilised irrespective of mussel sex. Entry of the female ovipositor through the mussel exhalent siphon is via three openings, one each side for the two outer gills and one central opening that subsequently splits into the two inner gills. Kitamura's (2006) study suggested an ability by females to select which gill set they place their eggs. Further research into how bitterling are able to detect the presence of glochidia and select which gills to use for oviposition would add another level of understanding to the already complex oviposition decisions of bitterling (Smith et al., 2000a; 2001; 2007; Reichard et al., 2007a; Phillips et al., 2017).

4.5.2 Lateral Choice

There was no evidence from the present study to support the idea that *R. amarus* display consistent handedness in selection of left or right gills, or for population effects.

While there is clear density-dependent selection in oviposition choice of bitterling (Smith et al., 2001), left/right morphs appear to not have evolved as a mechanism to reduce embryo mortality. While handedness polymorphs have been demonstrated in some taxa; in the case of bitterling, it appears that females may be able to adaptively allocate eggs flexibly into different regions of the mussel gill. This flexibility would be much more advantageous in limiting embryo mortality than the hypothesised consistent handedness morphs. The capacity to select 'microhabitats' for oviposition has been demonstrated in parasitoid wasps (Vet & van Opzeeland, 1984) and lizards (Angilletta et al., 2009), in the former case associated with host specificity and in the latter selecting an appropriate temperature for egg development. Conditions for embryos can change rapidly across an oviposition site, particularly when multiple clutches are places on the same patch or host. Avoidance of superparasitism is particularly important in the oviposition decisions of some insects and amphibians (Refsnider & Janzen, 2010) and, like bitterling, these taxa similarly utilise host/patch cues to select sites that maximise the success of offspring development.

4.5.3 Conclusions

It is unclear whether bitterling can actively select different gills or gill regions to avoid not just glochidia, but also conspecific eggs and embryos. While the pattern of oviposition suggests an equal distribution of eggs between left and right gills (Table 4.1, Figure 4.2), the detection of glochidia and conspecific eggs inside the gills may utilise specific cues that can be evaluated by the female during mussel inspection (**Chapter 2**). Further research will be needed to address these questions.

5.0 Chapter Five

INTER-POPULATION COMPARISONS SHOW MALE EUROPEAN BITTERLING TO BE SENSITIVE TO SPERM COMPETITION RISK AND PROBABILITY OF OVIPOSITION

5.1 ABSTRACT

Sperm competition occurs when the spermatozoa of two or more males compete to fertilise the same eggs. In some taxa, males perform multiple ejaculations, which may function in sperm competition or in maintaining a baseline density of spermatozoa in the female reproductive tract to ensure fertilisation, a process termed 'topping up'. The European bitterling (*Rhodeus amarus*), a small freshwater fish that fertilises and incubates its eggs within a freshwater mussel host, was used to investigate the reproductive function of mussel inspection and multiple ejaculation. Males from three populations, with different densities of fish compared to mussels, were exposed to either an additional rival male or a gravid female. The hypothesis was that if a male exhibited a greater response to the presence of rival males than a gravid female, then multiple ejaculations functioned primarily through sperm competition, and *vice versa*. Males increased the frequency of mussel inspection in response to the presence of a gravid female. Males from a population with high levels of mean crowding also showed an elevated frequency of mussel inspection and increased probability of ejaculation. These findings indicate a primary role of multiple ejaculations for 'topping up' of mussels with sperm in the mating system, but also suggest some role for sperm competition.

5.2 INTRODUCTION

5.2.1 Multiple Ejaculation Theory

Mating can potentially involve multiple ejaculations from one or several males, though the role of multiple ejaculatory behaviour in the context of sperm competition theory is equivocal (Parker, 1984; 1998; Smith & Reichard, 2013; Smith et al., 2014a). Two functions of multiple ejaculations have been proposed. First function is as a sperm competition behaviour. Multiple ejaculations may be more effective in competing with other males than a single large ejaculate, particularly if sperm competition risk is high (Parker, 1984; 1998). Second, in cases where there is passive loss of spermatozoa from the female reproductive tract or spermatozoa function, multiple ejaculations may be adaptive if males 'top-up' or replenish their sperm in the reproductive tract of a female (Parker, 1998).

5.2.2 Rhodeus amarus

Multiple ejaculations are a feature of the mating system of bitterling fishes (Smith et al., 2009; Smith & Reichard, 2013; Smith et al., 2014a). Males repeatedly release sperm over the inhalant siphon of the mussel but it is unclear whether this is to better compete with rival sperm or to ensure fertilisation of eggs within the mussel or perhaps both.

Prior to spawning, bitterling of both sexes perform 'inspection' behaviour of the exhalant siphon of a selected mussel (Wiepkema, 1961). Female inspection functions in assessing mussel quality as an oviposition site, with females responding positively to mussels with a high level of dissolved oxygen emerging from its gills; a reliable predictor of egg and embryo development rate and survival (Reichard et al., 2009; Phillips et al., 2017; **Chapter 2**). In males, the function of mussel inspection behaviour

during oviposition is less well tested. Male inspection behaviour may function in assessing the quantity of a male's own sperm in a mussel. Notably, males are responsive to the flow rate of water emerging from a mussel gill and respond to high flow rates with elevated rates of ejaculation (Phillips et al., 2017; **Chapter 2**). Sperm released into a mussel potentially undergoes passive loss from the gills as the mussel filters and expels water. Smith & Reichard (2013) proposed that because mussels filter water at different rates (either due to species or individual differences) males might be sensitive to mussel flow rate and should respond to elevated flow rates by increasing ejaculation rates to keep mussels 'topped-up' with their sperm (*sensu* Parker, 1998) to maintain a baseline level in a mussel gill, with mussel inspection behaviour permitting males to monitor sperm levels in a mussel. Thus, multiple ejaculations and male mussel inspection behaviour may be selected primarily through sperm competition. Alternatively, they may be adaptions for monitoring and 'topping up' mussels with sperm. These alternative hypotheses for male behaviour, which are not mutually exclusive, have yet to be tested.

Mussels are essential for bitterling reproduction, but can vary in abundance by orders of magnitude among populations (Smith et al., 2000a; Smith, 2017) leading to differences in 'mean crowding' (*sensu* Lloyd 1967). In the context of sperm competition, males exposed to high levels of mean crowding for fertilisations will experience a concomitant elevated risk of sperm competition and likelihood of spawning.

5.2.3 Hypotheses

The aim of this study was to understand the function of multiple ejaculations and male mussel inspection behaviour in *R. amarus*. The behaviour of males exposed to

either an additional rival male (elevated risk of sperm competition) or a female in spawning condition (elevated probability of oviposition) was examined. These experimental treatments were tested in three populations with different levels of mean crowding. In the case that selection for multiple ejaculations is primarily through sperm competition, an elevated frequency of ejaculations and mussel inspection behaviour was predicted when focal males were exposed to an additional rival, since this treatment imposes an increased risk of sperm competition. It was further predicted that an increase in both behaviours in populations with higher mean crowding was due to an elevated risk of sperm competition in populations with high levels of crowding. When exposed to a gravid female, no increase in the frequency of ejaculations or mussel inspection, and no response to crowding was predicted. In the case that selection for multiple ejaculations arises primarily through topping up, no response was predicted in the frequency of ejaculations or mussel inspections in response to an additional rival, and no effect of crowding. The suggested function of topping up is simply to maintain a baseline quantity of sperm in the gill of a mussel, irrespective of competition with other males. However, an elevated frequency of ejaculations and mussel inspection behaviour was predicted when focal males were exposed to a gravid female, with a stronger response in populations of higher mean crowding where the likelihood of oviposition is greater. This response is predicted because the baseline quantity of sperm a male maintains in a mussel should be sensitive to the likelihood of oviposition (Smith & Reichard, 2013). The two hypotheses are not mutually exclusive and could both play a role in multiple ejaculations. Predictions are summarised in Table 5.1.

Table 5.1. Study predictions for frequency of mussel inspection and ejaculation if male European bitterling behaviour is selected primarily through sperm competition or 'topping up'. ↑ indicates elevated frequency, →← indicates no response. Size of arrow indicates relative strength of response.

Treatment (interpretation)	Male rival (increased intensity of SC)			Gravid female (increased likelihood of oviposition)		
Population (crowding)	Ballica (high)	Kyjovka (low)	Sapanca (low)	Ballica (high)	Kyjovka (low)	Sapanca (low)
Selection through sperm competition						
Inspection	↑	↑	↑	→←	→←	→←
Ejaculation	↑	↑	↑	→←	→←	→←
Selection through 'topping up'						
Inspection	→←	→←	→←	↑	↑	↑
Ejaculation	→←	→←	→←	↑	↑	↑

5.3 METHODS

5.3.1 Study populations

Three populations of *R. amarus* were used for this study. Two study populations were located in Turkey, one from Lake Sapanca and the other from the River Ballica. A further population was located in the Czech Republic, in the River Kyjovka. These three populations are genetically distinct. The Ballica population experiences a high density of fish compared to mussel density, leading to a high mean crowding (Lloyd, 1967). Sapanca and Kyjovka experience low mean crowding (Casalini, 2013). Mussel

populations tend to be stable so this difference may have been present for 20 years or more (Smith, 2017).

5.3.2 Experimental animals

R. amarus from Lake Sapanca and the River Ballica were collected using baited fish traps in June 2013, at the peak of the *R. amarus* spawning season at these sites. Fish were transported to the University of Istanbul field station, Kurtköy, in aerated barrels and stored in aquaria measuring 700 (length) x 300 (width) x 400 (depth) mm at low densities, with a sand substrate and aquatic plants as refuges. They were fed commercial fish granules twice daily and were exposed to a natural light:dark cycle of 15:9 h prior to experimental work. River Kyjovka fish were collected in May 2016 using an electroshocker modified for capturing small fish. Fish were transported to the aquarium facilities of the Institute of Vertebrate Biology in Brno in aerated barrels and stored in aquaria measuring 1200 (length) x 400 (width) x 400 (depth) mm at low densities, with a sand substrate and fed commercial fish granules. Water quality was maintained using external power filters and the natural light:dark cycle was 15.5:8.5 h.

Unio pictorum mussels were used as spawning sites in all experimental treatments. This species is common to all three study sites and is readily utilised by *R. amarus* for oviposition (Smith et al., 2004; Reichard et al., 2010). In Turkey, mussels were collected by hand from the River Maşukiye in the Lake Sapanca basin (Ercan et al., 2013). Mussels for the Kyjovka population were collected by hand from an oxbow lake adjacent to the River Kyjovka. Mussels were collected before the bitterling spawning season and stored for the 5-week experimental period in an outdoor pool and fed with phytoplankton.

5.3.3 Experimental protocol

Two randomly selected males from the same population were placed in an experimental tank measuring 500 (length) x 400 (height) x 300 mm (depth) along with a *U. pictorum* mussel in a sand filled cup and artificial vegetation, and allowed to settle for at least 1 hour. The larger male became territorial around the mussel and was designated as the focal male. The smaller, non-focal male was retained in the aquarium throughout to encourage natural guarding behaviour by the focal male. The non-focal male was kept away from the mussel by the focal male and did not engage in ejaculations during observations or directly interfere with focal male behaviour, though its presence will presumably have had the effect of increasing the perceived risk of sperm competition.

Once the focal male began displaying mussel-orientated behaviours, one of two treatments was randomly imposed: either exposure to a gravid female or an additional rival male. In the gravid female treatment, a female with an extended ovipositor from the same population as the focal male was gently removed from a stock tank and confined in a 500 ml flat-sided glass box with a mesh top to allow water flow. The box containing the female was placed at the opposite end of the experimental tank to the mussel. Once the focal male commenced display behaviour to the female, the frequency of male inspection of the mussel exhalant siphon and ejaculation over the inhalant siphon of the mussel were recorded for 10 minute. After this time the focal male, non-focal male and female were captured and the standard length of each fish was measured to the nearest 1 mm. The total length of the experimental mussel was also recorded to the nearest 1 mm. No fish or mussels were used more than once in the study.

In the case of the additional rival male treatment, an identical experimental protocol was followed but with a mature male, rather than gravid female, presented in

the glass box. The same protocol was followed for all three experimental populations. Fifteen males in each treatment combination for three populations were tested, with a total of ninety focal males tested overall. There were no mortalities among experimental animals and after completion of the study all experimental fish and mussels were returned to their original site of collection.

5.3.4 Statistical analysis

To examine the effects of treatment (gravid female or additional rival male) and mean crowding (Ballica, Kyjovka, Sapanca) on territorial male response, two GLMs (Generalised Linear Models) were fitted to the data: one to model frequency of mussel inspection, the other to model ejaculation. Prior to analysis, a data exploration was carried out according to Ieno & Zuur (2015). Data were examined for outliers in the response and explanatory variables, homogeneity and zero inflation in the response variables, collinearity between explanatory variables and the nature of relationships between the response and explanatory variables were also examined. Ejaculation data were found to be zero inflated.

For mussel inspection data, a single outlier contributed to significant overdispersion of the data and was excluded from the analysis. There was no collinearity among covariates, and no evidence of zero inflation. The data were modelled using a negative binomial Generalised Linear Model (GLM), specified as:

$$Inspection_i \sim NegBin\ (\mu_i, k)$$

$$E(Inspection_i) = \mu_i$$

$$\text{var}(Inspection_i) = \mu_i + \alpha \times \mu_i^2$$

$$\log(\mu_i) = \eta_i$$

$$\eta_i = fPop_i + fTreat_i + msl_i + ssl_i + mtl_i$$

$Inspection_i$ is the number of mussel inspections by focal male i, which was assumed to follow a negative binomial distribution with mean μ_i and variance $\mu_i + \alpha \times \mu_i^2$, with a log link function. fPop_i is a categorical covariate with three levels, corresponding with population; Ballica, Kyjovka, Sapanca, f$Treat_i$ is a categorical covariate with two levels; either gravid female or additional rival male. The model also contained a linear effect for focal male standard length (*msl*), stimulus fish standard length (*ssl*), and mussel total length (*mtl*). This model therefore models inspection of a mussel by male bitterling in this experiment according to their population, the treatment applied to them (presence of male or female), and the size of the stimulus fish, the mussel and themselves.

Ejaculation data included two outliers and were significantly zero inflated. To address these problems the data were fitted using a Bernoulli GLM, which took the form:

$$Ejaculation_i \sim Binomial(\pi_i)$$

$$E(Ejaculation_i) = \pi_i$$

$$\text{var}(Ejaculation_i) = \pi_i \times (1 - \pi_i)$$

$$\text{logit}(\pi_i) = \eta_i$$

$$\eta_i = \text{f}Pop_i + \text{f}Treat_i + msl_i + ssl_i + mtl_i$$

$Ejaculation_i$ is the probability of ejaculation for male i assuming a Bernoulli distribution with mean π_i and variance $\pi_i \times (1 - \pi_i)$. The logit function ensures the fitted probability of ejaculation falls between 0 and 1. This models the probability of ejaculation by male European bitterling in this experiment, according to the population of the male, the

treatment applied (presence of male or female) and the size of the stimulus fish, the mussel and themselves.

5.4 RESULTS

The frequency that focal males inspected mussels was significantly higher for those from the River Ballica population than those from either the River Kyjovka or Lake Sapanca (Table 5.2, Figure 5.1). The mussel inspection rate of Kyjovka and Sapanca males did not differ (Figure 5.1). Male mussel inspection frequency differed significantly between experimental treatments, with higher inspection rates, irrespective of population, when focal males were exposed to a gravid female. There were no statistically significant effects of the size of the focal male or mussel on inspection frequency, though there was a marginally significant effect of the size of the stimulus fish (Table 5.2, Figure 5.1).

Table 5.2. Parameter estimates for mussel inspection frequency by male European bitterling modelled using a negative binomial GLM. Bold text indicates $P < 0.05$.

Model parameter	Estimate	s.e.	*z*	*P*
Fixed intercept$_{(Ballica)}$	6.49	2.20	2.95	**0.003**
population$_{(Kyjovka)}$	-1.09	0.34	-3.24	**0.001**
population$_{(Sapanca)}$	-1.15	0.33	-3.50	**<0.001**
treatment$_{(male)}$	-0.65	0.22	-2.96	**0.003**
focal male SL	-0.03	0.02	-1.29	0.196
stimulus fish SL	-0.06	0.03	-2.06	**0.039**
mussel TL	0.00	0.02	0.04	0.966

Probability of ejaculation was greater for focal males from the Ballica population than the Sapanca population, though there was no statistically significant difference between the Ballica and Kyjovka populations or Sapanca and Kyjovka populations (Table 5.3, Figure 5.2). There was no statistically significant effect of experimental treatment on male probability of ejaculation, size of focal male, stimulus fish or mussel (Table 5.3, Figure 5.2).

Table 5.3. Parameter estimates for ejaculation frequency by male European bitterling modelled using a Bernoulli GLM. Bold text indicates $P < 0.05$.

Model parameter	Estimate	s.e.	*z*	*P*
Fixed intercept$_{(Ballica)}$	5.15	5.70	0.90	0.367
population$_{(Sapanca)}$	-2.16	0.96	-2.26	**0.024**
population$_{(Kyjovka)}$	-1.26	0.83	-1.52	0.128
treatment$_{(male)}$	-0.77	0.56	-1.39	0.166
focal male SL	-0.09	0.06	-1.46	0.145
stimulus fish SL	-0.13	0.08	-1.63	0.103
mussel TL	0.06	0.05	1.25	0.211

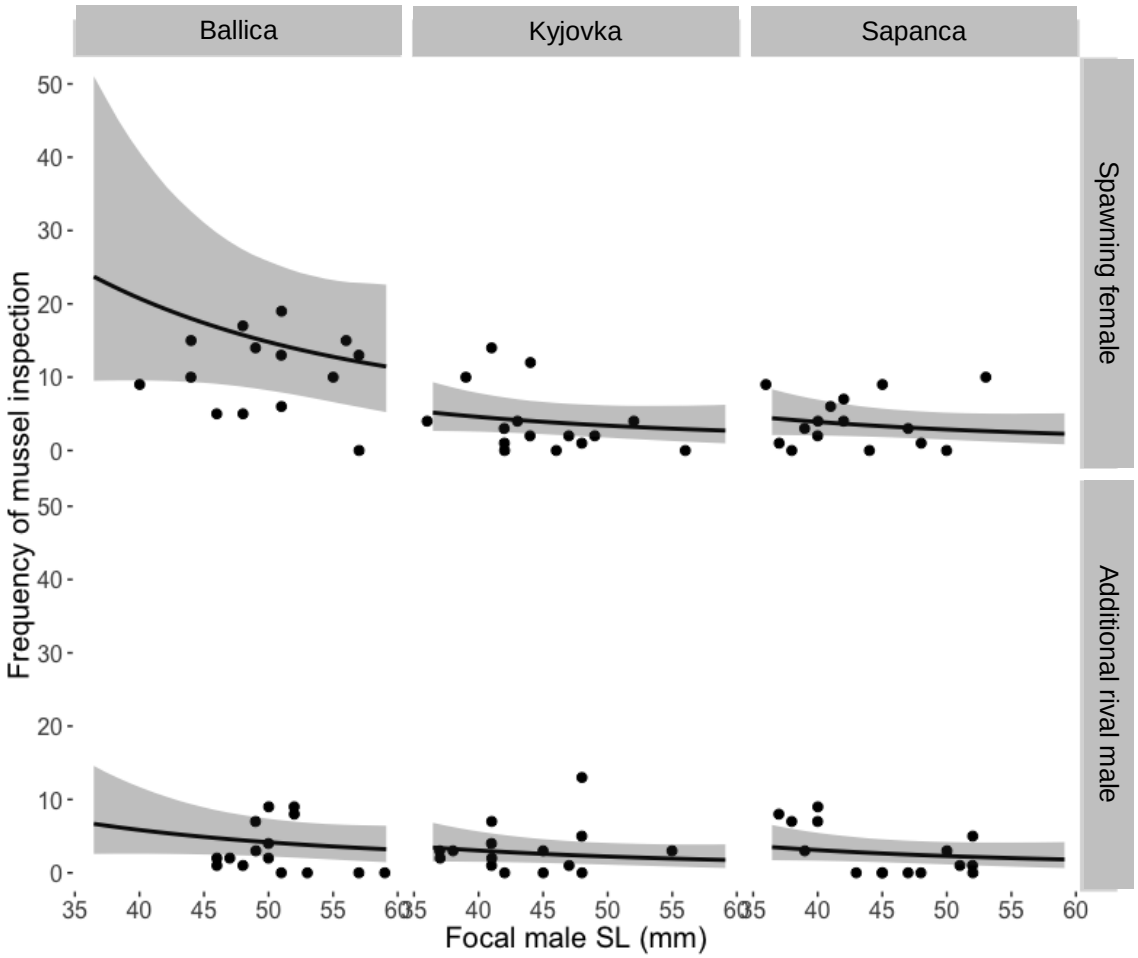


Figure 5.1. Mean fitted frequency (solid line) of mussel inspection behaviour by European bitterling (*Rhodeus amarus*) and 95% confidence intervals (shaded area) for males from three populations (Ballica, Kyjovka, Sapanca) and two experimental treatments (exposure to a gravid female, exposure to an additional rival male) for a negative binomial GLM. Black circles are observed data.

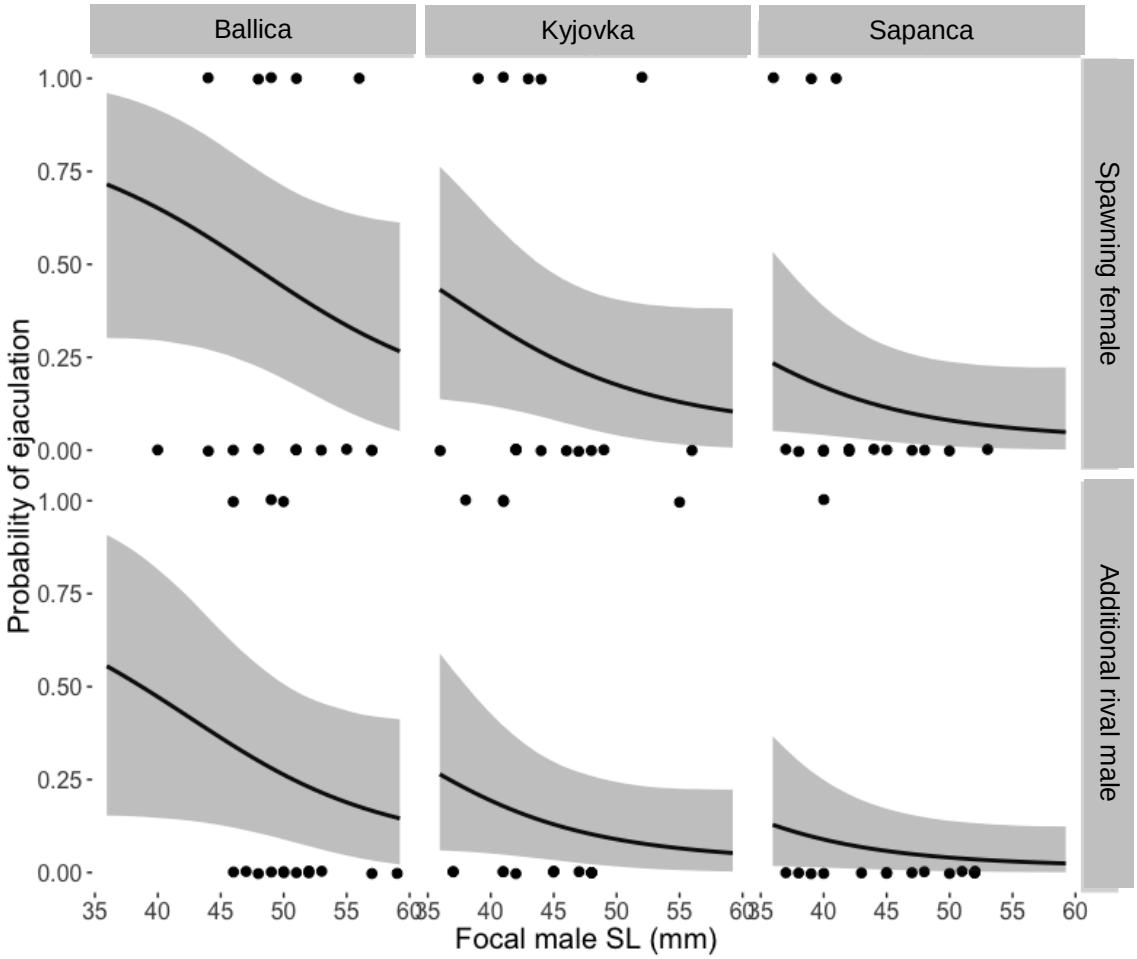


Figure 5.2. Mean fitted probability (solid line) of ejaculation by European bitterling (*Rhodeus amarus*) and 95% confidence intervals (shaded area) for males from three populations (Ballica, Kyjovka, Sapanca) and two experimental treatments (exposure to a gravid female, exposure to an additional rival male) for a Bernoulli GLM. Black circles are observed data.

5.5 DISCUSSION

The aim of this study was to examine the responses of male *R. amarus*, from different populations with different levels of mean crowding (*sensu* Lloyd, 1967), to either a rival or gravid female, to understand the function of mussel inspection behaviour and

multiple ejaculations. Experimental outcomes most closely matched predictions for a role of topping up in male behaviour (Table 5.1).

5.5.1 Male Inspection

The function of mussel inspection behaviour by male bitterling has hitherto been disputed (Smith et al., 2004). In females, there is a clear link between mussel-directed behaviours and choice of mussel as a site for oviposition (Smith et al., 2001; 2007; Phillips et al., 2017; **Chapter 2**). Earlier results (**Chapter 2**) unambiguously demonstrated male preferences for high flow conditions and specific odour cues in the water flowing from an artificial mussel, suggesting that flow may relate to sperm loss within mussel gills and therefore be a cue for sperm investment. Mussel inspection behaviour by males occurs frequently; the mean rate in the present study, irrespective of population, was 0.5 min^{-1}, which is comparable to that from other studies (e.g. Spence et al., 2013). Since males are territorial and guard the same mussel for extended periods, sometimes for several weeks (Smith et al., 2004), it seems unlikely that they need to assess mussel flow at this frequency. The results of the present study showed that inspection rates increased in response to a gravid female (Table 5.2, Figure 5.1), and were also higher in males from a population with high levels of mean crowding. The elevated response to female presence matches predictions for selection for mussel inspection behaviour through 'topping up' (Table 5.1).Given the importance of pre-oviposition ejaculation in the reproductive success of *R. amarus*, maintaining a baseline level of sperm in a mussel through multiple ejaculations and monitoring sperm density in the exhalant flow of a mussel through inspection behaviour may be critical to male reproductive success. The quantity of sperm in the gills of a mussel depletes through the action of filtration by the mussel (Smith & Reichard, 2013). Immediately following

ejaculation, the number of spermatozoa in the mantle cavity of a mussel increases rapidly, peaking after approximately 30 s. Thereafter the number of spermatozoa declines exponentially and after 10 minute the density of sperm is over an order of magnitude lower than at the peak (Smith & Reichard, 2013). However, the decline in sperm is unpredictable, with variation in ejaculate size, ejaculation frequency and mussel filtration rate all contributing to changes in spermatozoa density. Frequent monitoring of the quantity of mussel flow may allow a male to manage spermatozoa density in guarded mussels.

Attention to female presence was predicted to change with the probability of oviposition. This broadly fitted the data observed for male inspection behaviour. Stimulus fish size had a marginally negative effect on male inspection rate (Table 5.2, Figure 5.1), with smaller stimulus fish provoking a higher rate of inspection by focal males. In *R. amarus*, small males are more likely to mate using 'sneaky' mating tactics (Smith et al., 2009) and to engage in sperm competition (Reichard et al., 2004a; Spence et al., 2012). This finding implies focal males were more vigilant in examining guarded mussels in the presence of a smaller male and may relate to sperm competition evaluation.

5.5.2 Male Sperm Release

The ejaculation frequency of males among populations followed predictions for an increased risk of sperm competition (Table 5.1, 5.3, Figure 5.2), with the population with the highest mean crowding displaying the highest ejaculation rates. However, focal males did not respond to the presence of an additional male (Table 5.3). The additional male would represent an elevated *risk* of sperm competition (Parker, 1998), and was

predicted to elicit a greater frequency of ejaculations if the sperm competition hypothesis was correct.

Ejaculatory behaviour may function in the context of both sperm competition and 'topping up'. Male *R. amarus* are sensitive to the risk of sperm competition and modulate ejaculate expenditure to match that of rivals (Smith et al., 2003; Smith et al., 2014a; Table 5.3). Males are also sensitive to the presence of a gravid female (Table. 5.3) and modify their investment of sperm to maximise the likelihood of fertilisation (Smith et al., 2014a). These results implicate a role for both direct sperm competition and 'topping up' in the ejaculatory behaviour of *R. amarus*, as well as mussel inspection behaviour. Thus, the interpretation of these results is that mussel inspection has a role in topping up, perhaps in detection of sperm loss from a guarded mussel (**Chapters 2 & 3**). Elevated ejaculation and inspection rates in males from a population where mean crowding was greatest (Figure 5.1, 5.2), also implicates a role for sperm competition in male responses.

A feature of the experimental design was that a rival male was present in both experimental treatments to standardise effects and was required to stimulate the focal male to defend the experimental mussel. Thus, even in the gravid female treatment the focal male faced a baseline sperm competition intensity, which was elevated in the case of the additional rival male treatment. A possible reason for the failure to detect a statistically important treatment effect on ejaculation frequency may have been because there is a minimum sperm competition intensity threshold, represented by a single rival, above which males do not show any further marked response, though this is not expected to be the case (Parker, 1998; Smith & Reichard, 2013).

5.5.3 Population Differences

A caveat of our experimental design is that the difference between natural and experimental conditions may have affected the response seen in population treatments. This is an unavoidable aspect of using natural populations. With additional funding and time it would be possible to breed generations of bitterling in high and low mean crowding, allowing testing to occur and a middle crowding level, removing the different change in crowding conditions for different populations.

The differences in the frequency male inspection and ejaculation were observed among populations may be innate or learnt. All three bitterling populations were genetically distinct, including the Ballica and Sapanca populations (despite their geographical proximity), though they belong to the same phylogenetic clade (Bryja et al., 2010). The Kyjovka population occupies a different genetic clade to the two Turkish populations (Bryja et al., 2010). While few studies have examined the temporal stability of mussel populations, they appear stable over a time scale of at least 20 years (Smith, 2017), but possibly longer. Thus, given the genetic integrity of the bitterling populations in the study, and stability of mussel populations over at least a multi-decadal timescale, it is possible that *R. amarus*, which are annual fishes (Smith et al., 2000b), had sufficient time to genetically adapt to prevailing levels of mean crowding in each population. Common garden experiments, in which male *R. amarus* from different populations are raised in common conditions, are needed to further explore the plasticity of male mating behaviours.

5.5.4 Conclusions

In conclusion, male *R. amarus* from a population with a high level of mean crowding (*sensu* Lloyd, 1967) and that were exposed to a gravid female, inspected

mussel spawning sites at an elevated frequency compared to when presented with a male. The probability of ejaculation by males was similarly greatest for the population with the highest level of mean crowding, but did not vary when presented with either a female or additional male. These findings provide evidence that the function of mussel inspection behaviour by males is primarily to monitor guarded mussels in the context of 'topping up' with sperm.

6.0 Chapter Six

DEFINING MALE OVIPOSITION TACTICS IN THE EUROPEAN BITTERLING USING SOCIAL NETWORK ANALYSIS

6.1 ABSTRACT

Analysis of sperm competition in alternative mating systems can be complex when taking into account the possible interactions among individuals, particularly when males switch between mating tactics dependent on situation. Previous work on alternative mating tactics may have oversimplified cases where behaviours are highly flexible and males compete with rivals of differing size and tactics. The present study utilises a sperm competition social network for male European bitterling (*Rhodeus amarus*), a small freshwater fish that incubates embryos within freshwater mussels and exhibits alternative mating tactics. This novel method is used to identify traits such as size or sperm allocation that can be used to identify male tactics at a group level and to test if there are network characteristics for different male mating tactics. Using weighted network modelling, bitterling were allocated into mating categories on the basis of the distribution of sperm among oviposition sites. Further modelling identified a high degree of flexibility in males to switch between alternative mating tactics, supported previous work. Previous analysis of the bitterling mating system has typically assumed an individual-specific stable tactic for males. This network analysis allows for dependency between individual actions and flexibility in the tactics used by a single male, offering a fuller understanding of the complexity of this and other mating systems.

6.2 INTRODUCTION

6.2.1 Alternative Mating Tactics

Alternative male mating tactics evolve in a mating system when more than one reproductive mode or mating niche is possible (Wootton & Smith, 2015). Selection for alternative tactics is driven by sexual selection, and because variance in male reproductive success is typically higher in males than females, alternative tactics are typically associated with males (Shuster, 2010). Male mating polymorphisms have been investigated extensively (Godin, 1995; Cook et al., 1997; Denoël & Doellen, 2010) and often manifest as two main classes of traits under sexual selection, though other classes exist. Guarder males tend to be larger, more colourful, aggressive and, court females while driving away rivals. Non-guarders or sneaker males, tend to be small, passive, cryptic and, display no courtship. This pattern is exemplified in Teleosts by bluehead wrasse (*Thalassoma bifasciatum*) (Warner, 2001), bluegill sunfish (*Lepomis macrochirus*) (Neff et al., 2003) and Atlantic salmon (*Salmo salar*) (Gage et al., 1995). Terminology and specific behaviours vary among species, and in some taxa a female mimic phenotype has also been described (Taborsky, 2008). Average reproductive success may vary between tactics, with the guarder tactic typically gaining the highest reproductive success. In this case, non-guarder males can be considered as making the "best of a bad job". In other cases the reproductive success of each tactic may be equivalent, with the proportion of each mating tactic maintained by negative frequency-dependent selection (Gross, 1991; Dodson et al., 2013). The terms reproductive strategy and tactic are commonly used when discussing male mating polymorphisms. A male can display varied tactics under a more fixed strategy (Wootton, 1984; Wootton & Smith, 2015). Here the term tactic is used as it implies the appropriate degree of

flexibility in behaviour that best reflects the bitterling mating system (Smith et al., 2004).

As part of their oviposition decisions, males must make sperm allocation choices. Sperm is not an unlimited resource (Andersson, 1994) and intense sperm competition can result in male sperm depletion and reduced paternity success, leading to strong selection on male ejaculate expenditure (Parker, 1998). How males distribute their gametes is vitally important for understanding the mating system of a species.

Alternative mating tactics are typically associated with sperm competition (Andersson, 1994). Sperm competition occurs when two or more males attempt to fertilise the same eggs and is a key feature of mating system evolution (Parker, 1990; 1998). In the context of mating tactics, guarder males attempt to aggressively drive away rival males and monopolise matings, thereby reducing their risk of sperm competition. In contrast, non-guarders tend to compete with rivals primarily through sperm competition and cryptic behaviours or appearance.

Alternative tactics can be fixed or plastic over a male's lifetime. Fixed tactics are determined genetically and often characterised by striking morphological differences such as size and colour (Dominey, 1984). Plasticity in tactics can be sequential or reversible. In species showing sequential tactics, males will switch tactic at a threshold age, status or size and retain that tactic thereafter. For example, in the bluehead wrasse males switch permanently from initial phase to terminal phase based on body size (Gage et al., 1995; Warner, 2001). In the case of reversible tactics, males may opportunistically select between tactics according to the prevailing conditions though our understanding of tactic switching is poor (Smith et al., 2004; Wootton & Smith, 2015). Thresholds for sequential changes in mating tactics have been more

comprehensively studied and provide valuable information about the key attributes for male reproductive success. For example, salmonid early growth defines male tactic (mature parr or sea-migrating anadromous males), alongside an additive genetic effect (Heath et al., 1994; Foote et al., 1997). Thus, early growth can be an important predictor of male fitness, since the success of male tactics is often related to size at spawning (Gross, 1985). Further investigation of switching in reversible-tactic species offer a means of elucidating the key attributes of male reproductive success.

6.2.2 Rhodeus amarus

Bitterling lend themselves to experimental investigation of alternative mating tactics. Males play a significant role in oviposition choice by limiting spawning rates (Smith et al., 2000a) and influencing mussel choice (Smith et al., 2002; **Chapter 3**). A critical feature of the bitterling mating system is that guarder and sneaker males attempt to pre-empt female spawning by performing pre-oviposition ejaculations among multiple mussels. This behaviour permits investigation of how males make sperm allocation decisions to maximise fertilisation success against fertility assurance and sperm competition. While research on bitterling alternative mating tactics has highlighted the flexibility of the guarder/sneaker continuum (Režucha et al., 2012), most studies continue to address individuals as either guarder or sneaker and assume stable tactic choices, at least in the short and medium term (Reichard et al., 2004a; b).

A goal of the present study is to use social network analysis to characterise male mating tactics in the European bitterling to understand how males distribute ejaculates among oviposition sites in space and time. Social network analysis allows the modelling of the likelihood of ties between nodes (Whitehead, 2008; Croft et al., 2008). In this case, competing sperm releases linked males in a sperm competition network. Social

network analysis has been used extensively to investigate animal behaviour but has rarely been used in the context of mating system evolution and never as a tool to understand sperm competition (McDonald & Pizzari, 2016).

Social network analysis was used in the present study to test whether alternative mating tactics in European bitterling should be considered as two discontinuous behaviours or a continuous flexible response by males to changing social conditions. In identifying tactics used by experimental males, three measures were examined; male size, ejaculation per mussel inspection, and ejaculation per mussel visit. These were previously hypothesised as indicators of guarder or sneaker behaviour in bitterling (Mills & Reynolds, 2003; Casalini et al., 2010; Řežucha et al., 2012). Once tactics were designated to each male, the sperm competition network was modelled with the aim of identifying interactions of the assigned tactics.

6.3 METHODS

6.3.1 Experimental Setup

The study was conducted in a large outdoor concrete pool at the Institute of Vertebrate Biology, Brno, Czech Republic in April/May 2017. The pool measured 12.4 x 6.0 x 0.6 m. Mean (± s.e.) water temperature was 17.4 (± 0.1) °C over the course of the experiment. The pool was stocked with 12 male and 250 female *R. amarus,* captured by electrofishing in the River Kyjovka. Equal sex ratios were considered however time and water quality constraints required that the experiment be completed in two weeks. Limited numbers of females are gravid at any one time so a female-biased sex ratio ensured gravid females were always present during observations.

Fish were fed *ad libitum* with frozen chironomid larvae daily. Prior to stocking, males were measured to the nearest 1 mm and marked with coloured elastomer tags just below the dorsal fin, with two marks on each side of the body. Five colours of tag were used to produce twelve unique marks. Eight *U. pictorum* were placed in gravel-filled flowerpots (110 mm height and diameter) as spawning sites, spaced 2 m apart around three sides of the pool. One side of the pool was left free of mussels to enable observers to enter and leave the pool without disturbing experimental fish. Mussels were measured to the nearest 1 mm and replaced daily to control for a decline in mussel quality as ovipositions occurred in them (Smith et al., 2000a). Large plastic plants (0.7 m length) were placed around the margins of the pool as refuges. After stocking, fish were left for 2 days to settle before observations began.

6.3.2 Experimental protocol

Each mussel was observed by a snorkeler for 10 minutes, twice each day for 11 days. The frequency of inspection and sperm release by all visiting males were recorded along with male identity and mussel location. Subjective identification of male role was made by snorkelers and were defined over the trial observation period and from experience of observing bitterling behaviour in the wild (Table 6.1).

Table 6.1. Male role descriptions made by snorkers. These definitions were also combined to give the more common binary designation of territorial/guarder (territorial and pirate) and sneaker (resident, shoaling and independent sneaker) categories.

Role	Description
Territorial	A male stationed beside the mussel for the majority of the observation period and attempted to chase away males that approached.
Residential Sneaker	A male that consistently attempted sneak fertilisations, remained in the vicinity of a mussel for most of the recording period and taking over territorial behaviour for periods when the territorial male was absent
Shoaling Sneaker	A male that arrived at the mussel with a roaming group of females and attempted to sneak fertilisation, but with no attachment to the particular mussel.
Independent Sneaker	A male defined as a sneaker without site attachment, but one that also did not join a group of females.
Pirate	A male that arrived at the mussel, aggressively displacing the territorial male, and briefly defending the mussel and courting females. Pirates were often found to have an area of piracy that they patrolled between 3 or 4 mussels.

6.3.3 Statistical analysis

Data were analysed using the *statnet* (Handcock et al., 2008) and *network* packages (Butts et al., 2008) in R (R Development Core Team, 2017). Preliminary dynamic network modelling suggested that the network was still changing during the first two days of data collection and data for these days were subsequently dropped from the analysis (Luke, 2015). The 'gambit of the group' (Whitehead & Dufault, 1999) assumes associations between individuals by shared group membership and can be controversial, usually requiring heavy filtering to ensure that only true associations are used in analysis. Thanks to the experimental design, no 'gambit of the group' or filtering was required as direct observations of behaviours were made and there was no uncertainty

about male identity. The *ergm* package (Handcock et al., 2017) was used to model the weighted network.

The three measures of male tactic (male size, ejaculation/inspection ratio and ejaculation/mussel ratio) were applied as node attributes to the corresponding males in three models. The fit of each subsequent model was compared by Akaike Information Criterion (AIC) (Akaike, 1973) to identify the measure that best fitted the data. While eight mussels were present in the experiment, territorial males would defend multiple mussels within their territory, hence why only five males were designated as territorial.

Table 6.2. Table illustrating focal male identity, standard length, ejaculation/mussel ratio and designated tactic based on ejaculation/mussel ratio and subjective observation.

Male identity	standard length (mm)	ejaculation/ mussel ratio	male tactic designation by e/m ratio	male tactic designation by observation
blueblue	50	4.3	territorial	territorial
bluered	46	3.0	sneaker	sneaker
bluewhite	47	5.2	territorial	territorial
greengreen	48	7.7	territorial	territorial
greenwhite	48	7.6	territorial	territorial
orangeblue	46	6.8	territorial	sneaker
orangeorange	46	8.4	territorial	sneaker
redblue	45	2.6	sneaker	sneaker
redorange	42	3.7	sneaker	sneaker
whitegreen	55	8.0	territorial	territorial
whiteorange	44	3.3	sneaker	sneaker
whitewhite	45	8.4	territorial	sneaker

A weighted network model was constructed containing the appropriate terms for the effects of tactic and size. These included; sum, *nodematch*, *nodecov*, *absdiff* and *mutual*. Sum is the intercept of the model, *nodematch* tests if males of a similar variable (such as ejaculation to mussel ratio) compete more, *nodecov* tests the overall effect of a variable, *absdiff* ('size') tests how the difference in body size between node impacts on the likelihood of sperm competition, and *mutual* tests the likelihood of reciprocation of sperm competition. A best-fitting model was identified using AIC:

Likelihood of Tie ~ Sum + Nodecov('Ejac/Muss') + mutual

This weighted network model identifies the likelihood of two males in this experiment competing in sperm competition. The model has two covariates, the spread of the male's ejaculations across multiple mussels (Ejac/Muss) and how the release of sperm may cause the rival male to release more or less sperm in response.

6.4 RESULTS

Ejaculation/mussel ratio provided the best-fitting model of the three measures of male mating tactic. The ratio encompassed a spectrum of 2.6 to 8.4 ejaculations/mussel (Table 6.2) and was used to designate a male's tactic as territorial or sneaker. This ratio was compared to the subjective designation by observers during the study to confirm the model was correctly identifying male tactics and to designate males with a higher ejaculation/mussel ratio as those that use the sneaker tactic more often (Table 6.2).

More territorial males (as defined by higher ejaculation per mussel ratio) were more likely to compete in sperm competition than sneaker males, indicated by positive *nodefactor*('E/Muss') estimates (Table 6.3). Negative mutuality estimates (Table 6.3) indicated that males did not reciprocate sperm competition. If a male releases sperm

repeatedly into a mussel, other males are less likely to compete. A simulation based on the above model demonstrates the interconnectedness of the system, with each male competing with every other male at least once during the observation periods (Figure 6.1)

Table 6.3. Summary table of weighted Bayesian network model of the sperm competition interactions of 12 *R. amarus* males over 9 days. The variable *Sum* acts as an intercept in weighted modelling. Bold text indicates $P < 0.05$.

Variable	Estimate	s.e.	*P*
Sum	1.18	0.155	**<0.001**
Nodecov('E/Muss')	0.0872	0.0122	**<0.001**
mutual	-0.108	0.0879	**<0.001**

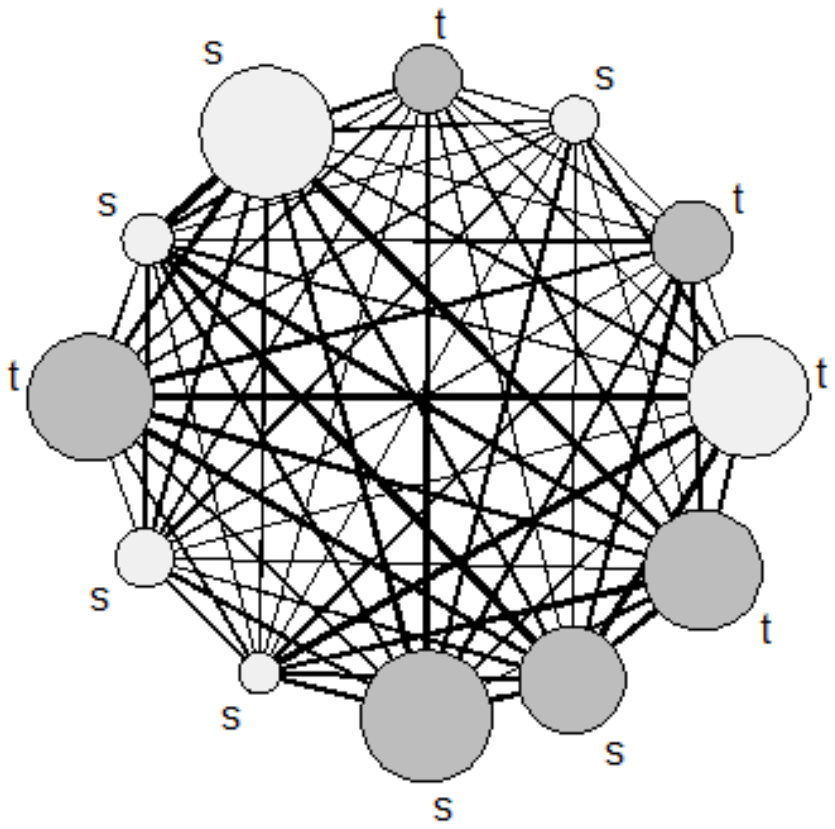


Figure 6.1. Illustration of sperm competition network among 12 *R. amarus*. Label and node colour indicate subjectively-designated mating tactic (t = territorial/guarder, s = sneaker). Node size is proportional to ejaculation/mussel ratio and tie thickness represents the number of sperm competition interactions; i.e. overlapping ejaculates in the same mussel during a 10-minute observation period. Node location confers no information.

6.5 DISCUSSION

The aim of this study was to use social network analysis to characterise male mating tactics in the European bitterling and understand how they distribute sperm among multiple oviposition sites. The network modelling demonstrated a positive effect of ejaculation to mussel ratio on sperm competition experienced by a male and a negative effect on reciprocation (Table 6.3, Figure 6.1).

6.5.1 Identifying Male Tactics

Results showed that male *R. amarus* are flexible in their mating tactics along a continuum (the rate of sperm release across mussels) rather than males displaying discrete, discontinuous behavioural phenotypes (Foote et al., 1997; Wootton & Smith, 2015). Size, ejaculation/mussel ratio and ejaculation/inspection ratio all displayed a range of values without a distinct cut-off between tactics. Ejaculation/mussel ratio fitted the data best of the three measures tested (Table 6.3), though all showed a similar pattern of tactic use in most males.

Males that released more sperm in fewer mussels were best defined as territorial and this conclusion was supported by subjective observations during the experiment. This finding suggests that the best way of characterising bitterling male tactics is through the distribution of ejaculates among oviposition sites, which permits an objective and quantifiable spectrum of behaviour along which males are more likely to perform a particular tactic.

6.5.2 Male Network Attributes

Network analysis has not previously been used to analyse sperm competition among males but has proved effective, allowing a comprehensive and objective analysis of male-male interactions without many of the problems typically encountered by network analysis (such as the gambit of the group) (Whitehead & Dufault, 1999). The best fitting model (Table 6.3, Figure 6.1) suggested that those males that invested more per mussel (displayed more territorial-like behaviours) were more likely to experience a greater intensity of sperm competition. This finding accords with Řežucha et al.'s (2012) findings on personality traits in European bitterling, which demonstrated that territorial males had relatively larger teste size than sneaker males and matched Parker

et al.'s (1996) model predictions. Thus, males initially match rival male sperm investment but reduce investment per rival as more rivals compete (Candolin & Reynolds, 2002; Smith et al., 2003). As territorial males must compete against multiple males (at least in the bitterling mating system), selection favours greater male investment in testis size. The network model also demonstrated a negative effect of mutuality between males. This negative effect indicates that if one male releases sperm repeatedly, other competing males release less sperm and do not try and match sperm releases. . Together, these findings describe a small number of territorial males invest heavily in ejaculates in a two or three mussels, while other males ("sneakers") competed at lower levels across a wider range of spawning sites. This differs from the often tested lab conditions of one mussel and two males (Reichard et al., 2004a; b).

Despite previous research showing that size is a critical trait in bitterling dominance (Kanoh, 2000; Smith et al., 2002; Reichard et al., 2005; 2009; Casalini et al., 2009), the best-fitting model did not predict size to be a male trait that contributed to the structuring of the sperm competition network (Table 6.2, 6.3). Many previous studies have been lab-based and investigated competition on a one-to-one basis, while the present study examined 12 males interacting simultaneously. If only size contributed to dominance, the largest males would be expected to hold territories and the remaining males would adopt sneaking tactics. The conditions used in this experiment resulted in males claiming territories with multiple mussels, however it is not possible for these males to defend multiple mussels at all times. This may give smaller, opportunistic males partial access to territories from which they are repeatedly displaced. Other possible confounding factors include mussel quality and male condition. Mussel quality was kept constant during the present study by daily replacement with the same mussel

species that contained no bitterling embryos (Smith et al., 2001). However male condition could not be experimentally controlled and was not measured. Fish were fed to satiation daily throughout the study, though they may have entered the study in different condition. One male did develop an infection over the course of the experiment, though this individual continued to compete effectively with other males and there was no strong justification for removing the male from the analysis.

Subjectively designating male mating tactics by direct observation generated a wide variety of male tactics. The pirate tactic has been suggested previously in bitterling and a cichlid species (Ota & Kohda, 2006). Pirate behaviour was observed in the two largest males in the present study, with these males displacing rivals as they patrolled a large area containing multiple mussels. However, from a functional perspective these males did not differ from a territorial/guarder tactic. Measuring male reproductive success using paternity analysis alongside spatial analysis would provide a fuller picture of the adaptive outcome of different male tactics.

6.5.3 Conclusions

Previous studies using bitterling, while recognising flexibility in male mating tactics, have assumed stable tactics throughout a given study period. The present study demonstrates that this is an oversimplification, with even the largest males switching between tactics multiple times within the space of a single 10-minute observation period. The study also suggests that ejaculation distributed among oviposition sites is an important feature of alternative mating tactics. The study provides evidence that an objective variable to quantify and define male mating polymorphisms in the framework of social network analysis offers a powerful approach to defining a mating system. This method of examining sperm competition as a network has many further avenues of

study. The quality and quantity of mussel hosts may can easily be controlled and use of PIT tags or cameras would allow more intense data collection. It is hoped that further questions of host choice and sperm competition can be tested in this semi-natural setting.

7.0 Chapter Seven

DISCUSSION

7.1 OUTCOMES

The goals of this thesis were to further the understanding of oviposition-site decisions using a model species, the European bitterling, while also expanding our understanding of the mating system of this species.

7.1.1 Chapter 1

In **Chapter 1** I undertook a comprehensive literature review of research on the bitterling mating system. This review summarises our understanding of the mating system to date, and provides a resource for researchers using the bitterling as a model system. The overriding theme across the review was the flexibility seen in bitterling among species and populations. The marked variation in behaviour, physiology and morphology, along with recent phylogenetic studies (Chang et al., 2014; Kawamura et al., 2014), lends itself to a phylogenetic approach to mating system evolution by mapping evolutionary relationships among bitterling species in response to relevant environmental variables. **Chapter 1** highlighted that oviposition cues are a key component for understanding bitterling oviposition decisions.

7.1.2 Chapter 2

Chapter 2 investigated the mussel host cues detected by bitterling. Despite previous studies, the proximate cues used by bitterling in making oviposition-site decisions had not been experimentally tested in a comprehensive study. Using an artificial mussel, the role of mussel odour, oxygen concentration and, water flow were tested in males and females. A sex difference in host preference cues was found. While both sexes responded positively to mussel odour, only females preferred mussels that

exhibited cues pertaining to embryo survival (dissolved oxygen concentration) while males responded to the outflow velocity from a mussel, possibly related to sperm competition. This latter result also provided clues as to the function of male inspection behaviour of mussels.

7.1.3 Chapter 3

Given the significance for male and female reproductive success of how sperm is distributed among mussels, an additional study to address the role of sperm cues in oviposition decisions. In **Chapter 3,** a study is presented that tested the response of males and females to the addition of sperm to mussels. No direct detection of sperm was found, however, the visual behaviour of the male elicited a response by females. Female skimming behaviour likewise increased the response of males. This study illustrated the significance of sperm investment by males for females, and what behavioural cues from females prompted ejaculatory behaviour by males.

7.1.4 Chapter 4

Chapter 4 examined potential gill microhabitat preferences of female *R. amarus* during oviposition. Given strong frequency-dependent selection mortality of eggs and embryos during development inside the mussel gill, selection for female oviposition 'handedness' to avoid repeating egg laying in the same gill area had previously been proposed but required experimental testing. No individual tendency to use the left or right gills of mussels was detected, though a pronounced likelihood to spawn into the inner gills was demonstrated. This finding supports previous work by Kitamura (2006) in a related bitterling species, which suggests that females avoid ovipositing in the outer gills which often contain developing glochidia.

7.1.5 Chapter 5

Chapter 5 presents a study investigating male social cues relating to oviposition choice by males, and the degree of variation in oviposition decisions among bitterling populations. Males were tested for responses to the presence of a female or an additional male. Fish from three populations that differing in 'mean crowding' were used to examine responses. Focal males increased the frequency of mussel inspections in response to the presence of a gravid female. Males from a population with high levels of mean crowding also showed an elevated frequency of mussel inspection and probability of ejaculation. These findings indicate a significant role for 'topping up' of mussels with sperm in the mating system, while also suggesting a role for sperm competition. This study identifies a function for male mussel inspection behaviour, which had hitherto been an enigma, and further demonstrates inter-population variation in sperm allocation tactics.

7.1.6 Chapter 6

Utilising a novel method for understanding mating system structure using social network analysis, in **Chapter 6** a study is presented that investigated how males interact to compete and allocate sperm across multiple oviposition sites on a mesocosm scale. The social network demonstrated explicitly that males are highly conditional in their choice of mating tactics with the ratio of ejaculations per mussel being the best indicator of mating system structure. Males that demonstrated a more territorial/guarder tactic experienced greater sperm competition and released more sperm in response. These results showed the mating system to be a more complex system than a stable binary territorial and sneaker that is often assumed.

7.2 SYNTHESIS

The question posed in this thesis was: what are the mechanisms of bitterling oviposition choice? The short answer to this is through attending to relevant mussel cues, but that social cues play an important role. These findings contribute to our understanding of the bitterling mating system, but also of oviposition decisions more generally, emphasising the significance of cues that have direct relevance for fitness and highlighting the important role of social cues. Parasitism of an invertebrate by a vertebrate is unusual. Other model systems in brood parasites and parasitoids similarly highlight a role of cues for offspring survival (Soler et al., 1999; Mackaurer et al., 1996). Host choice cues utilised by parasitoid wasps are comparable to the bitterling system, with a hierarchy of visual and olfactory cues with identify the value of a host for oviposition (Mackaurer et al., 1996). Brood parasites, for example cuckoos, select different host species on the basis of availability, length of breeding season and nest type (Soler et al., 1999). Cuckoo hosts are similar to mussels in that their rejection of parasites is positively correlated with length of sympatry (Davies & Brooke, 1989; Soler & Møller, 1990).

7.2.1 Integration with Refsnider & Janzen (2010)

The work described in this thesis is intended to draw on our current understanding of oviposition across a range of taxa. Refsnider & Janzen (2010) reviewed current research on oviposition-site decisions, suggesting multiple hypotheses for oviposition decisions that varied depending on taxa. These hypotheses suggest that oviposition decisions function not only in selecting sites that reduce offspring mortality, but also take into account the role of males, natal philopatry, changing offspring phenotype, and the suitability of the habitat for offspring and maternal survival. These hypotheses are not mutually exclusive and can be considered as a hierarchy of effects,

with levels of importance given to different cues depending on the mating system. The bitterling model fits into this framework while contributing to our understanding of oviposition decisions.

7.2.2 Predation Risk

Among amphibians, reptiles, birds and insects, oviposition cues relating to predation of offspring were identified as the most important variables on oviposition decisions (Refsnider & Janzen, 2010). This is not the case in the majority of fish species which instead attend to environmental cues relating to suffocation and temperature-dependent mortality. In some species, such as the three-spine stickleback (*Gasterosteus aculeatus*) there is a trade-off between anti-predation behaviour and fanning eggs to help oxygen exchange (Sargent & Gebler, 1980) suggesting both were still important aspects of clutch fitness in this fish. Bitterling have developed a mating system that requires no consideration of predation avoidance cues, since the hard shell of their hosts effectively protects the embryos during development.

7.2.3 Environmental Conditions

Oviposition decisions taken by female bitterling are primarily to select optimal conditions within the host mussels for offspring survival (Smith et al., 2001; 2002; 2003; Agbali et al., 2012; **Chapter 2**). Across fish species, the high oxygen requirement of eggs has led to a variety of oviposition strategies (Shang & Wu, 2004; Greig et al., 2007). The bitterling utilisation of mussel hosts is one such strategy. The American shad (*Alosa sapidissima*) preferentially lays eggs in high flow reaches of rivers to help reduce suffocation of eggs during development (Bilkovic et al., 2002). The longfinned goby (*Valenciennea longipinnis*) select from a range of tunnelled burrows, which best facilitated flow over the clutch (Takegaki, 2001), Environmental cues relating to

offspring survival are similarly important in other taxa and relatively widely studied (Leslie & Spotila, 2001; Davis, 2005; Doody et al., 2009). **Chapter 2** demonstrated responses by female bitterling to dissolved oxygen cues and by males to water velocity. The contrasting cues used by males and females raises the prospect of an intersexual conflict in oviposition-site decision making, with oviposition sites that maximise female fitness differing from those that maximise male fitness. This subject has yet to be explored in other taxa as an avenue for sexual conflict.

7.2.4 Conspecifics

A further important factor in oviposition decision making is the role of conspecifics. The sharing of an oviposition patch can have a negative or positive effect on offspring survival through competition for resources or reducing environmental effects (van Alphen & Visser, 1990). Parasitic wasps leave marks on parasitised hosts to avoid competition, as embryos sharing a host experience reduced fitness (van Alphen & Visser, 1990). In amphibians, superparasitism may occur preferentially to avoid predation (Rudolf & Rödel, 2005), or be avoided as early hatching individuals may cannibalise conspecifics (Matsushima & Kawata, 2005). Bitterling are similarly attentive to cues from conspecifics, and utilise oxygen cues to avoid mussels already containing embyros (Smith et al., 2002; **Chapter 2**).

7.2.5 Hierarchy of Cues

Many species utilise cues for oviposition in a hierarchy; when one cue is equal between sites, a secondary cue is used to distinguish between them (Hoffmann & Resh, 2003; Reich & Dowens, 2003; Refsnider & Janzen, 2010; Patten et al., 2011). This was first proposed in bitterling by Heschl (1989) and is supported by work presented in this thesis. **Chapter 2** explicitly demonstrated a hierarchy of female cues from odour to

oxygen content in mussel outflow. **Chapter 3** demonstrated a role of male ejaculations when mussel quality was controlled and **Chapters 5 & 6** illustrated how male behaviour during spawning changed with the environment (mussel availability and male interactions), which can have a knock-on effect on female oviposition choices. It is this use of a wide range of cues, in a structured hierarchy that allows oviposition choices to be expressed flexibly in response to a range of environmental conditions, while maximising offspring fitness by utilising multiple cues to select the optimal oviposition site.

7.2.6 Role of Males

In their review of oviposition-site decisions, Refsnider & Janzen (2010) proposed the role of male behaviour in female oviposition choice as a key area of further study. In internally-fertilising species, males may have minimal effects on female oviposition choice, however, in external fertilisers and where males build nests, the fitness effects of male behaviour and oviposition choice may be critical (Refsnider & Janzen, 2010). Studies of the comparative importance of female mate and host choice has been hampered by the difficulty in manipulating male quality independently of host or nest quality. Slagsvold (1988) and Sikkel (1995) accomplished this by manipulating nest position and algal thickness of nests in the pied flycatcher (*Ficedula hypoleuca*) and the garibaldi damselfish (*Hypsypops rubicundus*) respectively. These studies manipulated nest quality characteristics after males had built nests but before females arrived. They demonstrated female preference followed nest quality over male quality but that nest and male quality were linked, indicating a role of male quality via nest building. As an externally-fertilising species with oviposition sites of variable quality guarded by males, male bitterling behaviour was expected to influence female

oviposition decisions. **Chapter 2** demonstrated a difference in how males and females respond to mussel cues, while **Chapter 3** results illustrated that male ejaculatory behaviour impacts female oviposition decisions. **Chapter 5** showed that male multiple ejaculation behaviour is a function of both sperm competition and likelihood of oviposition and **Chapter 6** demonstrated that males differentially distribute their ejaculates across oviposition sites according to mating tactic. This sequence of studies illustrate that male oviposition choice and, therefore, female oviposition choice, is influenced by both immediate and long-term sperm competition and the male mating tactic. However, if females attend to male ejaculatory behaviour, it offers a mechanism by which males can influence female oviposition decisions. It also raises the possibility that females might be able to bias paternity towards specific males. It has already been established that females solicit additional males to participate in mating through 'skimming' behaviour (Smith & Reichard, 2005). Additional studies are needed to examine the extent to which female sensitivity to sperm cues gives them cryptic control over parentage. Refsnider & Janzen (2010) predicted a role for males in female oviposition decision making, however, it has been rarely illustrated. The bitterling model system has demonstrated an important role of male decision making in oviposition choice.

7.2.7 Male Inspection

Chapters 2, **3**, **5** and **6** all demonstrated a role of male mussel inspection behaviour for both 'topping up' mussels with sperm and sperm competition. Previously, the role of male mussel inspection was disputed. Studies of male host preference had shown an indifference to mussel "quality" defined by female choice (now known to relate to oxygen cues, **Chapter 2**), and so males were assumed to have no strong host

preferences (Casalini et al., 2013). In fact, males have clear mussel preferences (**Chapter 2**) with mussel inspection by males serving to monitor the extent to which mussels are 'topped up' with their sperm, which relates partly to mussel filtration rates. Ostensibly, 'topping up' appears a bitterling-specific feature of a mating system. However, the concept of 'topping up' was devised in the context of internal fertilisation (Parker, 1984), though no experimental studies have yet been conducted on the concept of topping up other than in bitterling (Smith & Reichard, 2013). Candidate taxa with internal fertilization that are suitable for research on topping up are those in which the risk of sperm competition is low, where conception is unpredictable, but where sperm deposited in the female reproductive tract depletes over time (Smith & Reichard, 2013).

7.2.8 Intraspecific Differences

An important aspect of oviposition research is the testing of geographically distant populations to investigate variation and consistencies in behaviour among populations, including investigation of behavioural plasticity (Refsnider & Janzen, 2010). Studies presented in **Chapters 3**, **4**, and **5** addressed this question, comparing and contrasting populations from Czech Republic and two environmentally different locations in Turkey. These studies demonstrated similarities in ejaculatory behaviour and mussel microhabitat preferences. An exception was in male sperm allocation in response to the presence of a gravid female. Here, males that experienced a high risk of sperm competition through elevated levels of mean crowding inspected mussels at a higher frequency and maintained a higher level of sperm within their guarded mussels than males from geographically distinct populations with different levels of crowding (**Chapter 5**). These studies illustrate a degree of consistency among populations, but

suggest that mean crowding may shape bitterling mating systems, particularly in relation to male responses to sperm competition and topping up.

7.2.9 Alternative Mating Tactics

Alternative mating tactics have been extensively studied (Taborsky et al., 2008), with territorial and sneaker males shown to have impacts on female reproductive success (Jones, 2001; Fu et al., 2001; Watters, 2005; Preston et al., 2005). While many of these studies have investigated fixed alternative mating tactics, reversible tactics are predicted to be more common (Wootton & Smith, 2015). The research presented here on bitterling provides further evidence for the significance of flexible alternative mating tactics (**Chapter 6**), and while not definitive, highlights an objective approach to classifying mating systems.

7.2.10 Methods used

The analyses used in this thesis extend from frequentist GLMs, to weighted network Bayesian dynamic models. The latter analysis, used in **Chapter 6** opens up opportunities for a fuller understanding of spatial and temporal patterns in sperm competition, in bitterling or other taxa. This approach has enormous potential for understanding systems that are otherwise too complex to model.

Through this thesis, the bitterling model system has illustrated concordance and contrast with the conclusions of Refsnider & Janzen's (2010) review. The importance of environmental cues across many fishes was supported by the bitterling attendance to cues relating to offspring survival. In contrast, the bitterling utilisation of mussel hosts sets this group apart from other taxa, leaving them without the risk of predation or any behaviours related to parental care. This specialisation of mating strategy illustrates the

importance of looking at behavioural decisions at a genus or species level to avoid generalisations that may lead to misinformation about mating systems.

7.3 CONCLUSIONS

1. The European bitterling, and bitterling species more generally, offer a powerful model system for understanding mating system evolution, and oviposition decisions more specifically.
2. Male and female both correspond and differ in oviposition cues to which they attend. Intersexual differences in oviposition cues implies different selection pressures on males and females, potentially generating intersexual conflicts over oviposition-site decisions.
3. The function of male mussel inspection behaviour is now closer to being identified, with a role in monitoring mussel filtration rate for 'topping up' mussels with sperm, but with no role in directly detecting rival sperm.
4. Both male and female bitterling utilised social cues during oviposition, and this represents an avenue for further research in other taxa. This included a role of males affecting female oviposition choices.
5. Both behavioural plasticity and consistencies were identified in genetically distinct populations of the same species, providing examples of flexibility and conserving of behaviours across a species range.
6. Research on alternative male mating tactics tends to over-simplify male roles by imposing artificial classifications. This research has highlighted a means of using continuous measures of reversible tactics to understand male reproductive success.

7.4 FUTURE RESEARCH

Four key extensions to the current research present themselves.

7.4.1 Male Inspection Behaviour

The first would be to expand on the role of male mussel inspection behaviour (**Chapters 2, 3** and **5**). While the role of inspection in identifying mussel odour and flow has been shown (**Chapter 2**), the high rate of inspection seen in natural and laboratory conditions suggests the role has further functions. Inspection may function in directing females to the exhalent siphon of the mussel or detection of egg release. This question lends itself to an experimental approach and would further contribute to answering a key question on bitterling reproductive behaviour.

7.4.1 Female Control of Egg Position

Related to mussel inspection behaviour is the question of how females allocate eggs to different gills within a mussel (**Chapter 4**). Two hypotheses exist: that females can direct their ovipositor into certain openings past the exhalant siphon (Kitamura, 2006), or that oviposition placement is random and the pattern of water flow generated by the mussel internally plays a role in directing the eggs into the inner gills and thereby preventing the bitterling eggs from competing with the glochidia. The ability to select microhabitats for embryos would illustrate remarkable adaptation and fitness variation even within a mussel.

7.4.3 Interspecific Studies

The bitterling fishes are a large, specious group. While much of the work on *R. amarus* and *R. ocellatus* has been applicable among species, interspecific studies such as those of Smith & Reichard (2005), Kitamura (2007) and Pateman-Jones et al. (2011) have proved illuminating in highlighting differences in behaviour and physiology

among species, and identifying features in the environment that drives selection for these differences. The role of interspecific competition for mussel hosts is an additional area that could provide key information for conservation of endangered bitterling species (Kitamura, 2007). In this context, understanding mussel host specialisation and generalism in bitterling remains an important, but unanswered question (Reichard et al., 2007a).

7.4.4 Sperm Competition Network Analysis

Chapter 6 outlines a novel analytical approach that may provide new avenues of research in understanding sperm competition. Using bitterling, an experimental approach that automated the recording of male location and visits to mussels, such as PIT tags, combined with paternity analysis, offers a powerful approach to understanding the role of social networks in shaping mating system evolution.

In summary, this thesis has investigated the host, sperm and social cues of oviposition in the European bitterling and further builds our knowledge of the bitterling mating system, allowing more effective use of this model species.

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